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myc Genes and Their Deregulation in Lung Cancer

Kalle Saksela

Department of Virology, University of Helsinki, 00290 Helsinki, Finland

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The discovery made in the late 1970s that the transforming sequences of oncogenic retroviruses (v-*onc*) isolated from a diverse group of animal tumors were originally derived from genomes of infected host cells led to the discovery of the cellular oncogenes (c-*onc*) [1]. When functioning under strict regulation in normal cells, these genes are called protooncogenes to distinguish them from their activated counterparts, deregulated or structurally mutated oncogenes. Proteins encoded by cellular oncogenes have been identified in most cellular compartments and also as secreted proteins [2]. An interesting group of cellular oncogenes are those encoding nuclear proteins. Nuclear oncoproteins are thought to have an important role in growth regulation by controlling gene activity in the nucleus and/or by directly regulating DNA replication [3].

This review focuses on the *myc* family of nuclear oncogenes, which now consists of three well-characterized members, the c-*myc*, N-*myc*, and L-*myc* genes. The current data on *myc* gene regulation and deregulation, *myc* proteins, and their possible functions are reviewed. The results from our laboratory on amplification of *myc* genes in human lung cancer and on L-*myc* and N-*myc* protein characterization are summarized.

IDENTIFICATION AND STRUCTURE OF THE *MYC* GENES

The c-*myc* gene was cloned by virtue of its close homology with the v-*myc* gene isolated from an avian myelocytomatosis virus MC29 [4–8]. The c-*myc* gene was found to be evolutionarily well conserved between different species [9–13]. Cross hybridization of the *myc* genes then allowed the isolation of a family of closely related genes. The *myc* gene family now consists of at least six apparently functional members, the c-, N-, L-, P-, R-, and B-*myc* genes, and one inactive pseudogene L-*myc* Ψ [14–17]. However, the gene cloned, partially sequenced, and termed B-*myc* by Ingvarsson et al. [17] appears to be identical with the sequence designated R-*myc* by Alt and collaborators [14] but distinct from the B-*myc* gene isolated by them (F. Alt, personal communication).

Among the members of the *myc* gene family, only the c-*myc*, N-*myc*, and L-*myc* genes have been characterized in detail, including the determination of their complete

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cDNA and genomic sequences and transforming potential [9,18–24]. The N-*myc* gene was first detected and isolated from human neuroblastoma cells in which it was amplified [15], whereas the L-*myc* gene was found as an amplified homologous sequence from human small cell lung cancer (SCLC) [16]. The overall genomic organization of the c-*myc*, N-*myc*, and L-*myc* genes is quite similar. They all span from 6 to 7 kilobases in the human genome and consist of three exons that contain long untranslated, yet evolutionarily conserved, sequences. In contrast to the protein-coding domains, these sequences are not particularly homologous between the different *myc* genes. The human c-*myc* gene has been mapped to the long arm of chromosome 8 (8q24) [25]. The human N-*myc* and L-*myc* genes reside in chromosomal locations 2p23–24 [26] and 1p32 [27], respectively.

In contrast to the structural similarities, considerable differences have been found in the promoters and RNA processing patterns of the *myc* genes. Transcription of the c-*myc* gene starts by differential use of two well-defined promoters, designated P1 and P2, located about 150 bp apart [9]. More recently, a third promoter (P0), giving rise to multiple transcription start sites, has also been described farther upstream of the c-*myc* gene [28]. N-*myc* transcription is initiated from numerous sites clustered around two promoters [29]. By contrast, L-*myc* transcription is initiated from a single site in the 5′ end of the gene [23].

N-*myc* mRNA has been shown to occur in two differentially spliced forms, which differ in their untranslated 5′ sequences [29]. Differential splicing has also been reported for c-*myc* transcripts initiated from the P0 promoter [28], whereas no variation has been observed in the removal of the two c-*myc* introns from transcripts initiated from the major promoters P1 and P2. By contrast, L-*myc* mRNA processing is very complex and by differential splicing and use of multiple polyadenylation sites gives rise to distinct mRNA classes with considerably different protein-coding potentials [23]. No such mRNA heterogeneity due to differential use of polyadenylation sites has been observed for the c-*myc* or N-*myc* genes.

ACTIVATION OF THE *MYC* GENES IN HUMAN TUMORS

The c-*myc*, N-*myc*, and L-*myc* genes have all been found to be amplified in some human tumors. Amplifications are associated with highly enhanced mRNA expression and abnormally high levels of the corresponding protein products [30]. In some cases c-*myc* gene amplification has been found to be accompanied with a genomic rearrangement, which might further increase the oncogenic potential of the amplified gene [31]. Amplifications of the c-*myc* gene have been shown in a wide variety of human tumors [30,32], whereas amplifications of the N-*myc* or L-*myc* genes appear to occur predominantly in a more restricted set of tumors. In addition to the sporadic amplifications in various tumors, c-*myc* amplifications are particularly common in cell lines of SCLC (see below) [16,33,34], in breast cancer [35], and in cervical carcinoma [36]. N-*myc* amplifications are common in neuroblastoma [15,37], SCLC [38], and retinoblastoma [39], which all have neural or neuroendocrine characteristics. More recently, examples of N-*myc* amplification have also been reported in other tumors, such as adenocarcinoma of the lung [40], astrocytoma [41], and rhabdomyosarcoma [42]. L-*myc* amplifications have so far been encountered only in SCLC [33,43,44].

In Burkitt lymphomas, the c-*myc* gene is consistently found to be affected by specific reciprocal chromosomal translocation involving some of the immunoglobulin loci

[45]. Different models for the molecular mechanism activating the c-*myc* gene in these translocations have been proposed [9,46–51], but the structural and functional basis of this phenomenon remains unsettled. Curiously, there are no examples of N-*myc* or L-*myc* gene activation by a chromosomal translocations.

The single-copy, nonrearranged c-*myc* gene is expressed at high or moderate levels in most malignant cells [52], but the underlying activation mechanisms are in most cases not understood. It may be that the high c-*myc* expression in many cases is only a consequence of malignant transformation, since c-*myc* expression is known to be associated with high cellular proliferative activity in general (also in normal cells) (see below). On the other hand, high levels of expression of the c-*myc* gene may also be due to stimulation by autocrine growth factors, known to be produced by many tumor cells [53]. In fact, the only malignant cells identified so far that do not express any c-*myc* mRNA have been cells with another activated *myc* gene [44,54,55], suggesting some kind of cross-regulation between the *myc* genes. In contrast to c-*myc*, high N-*myc* or L-*myc* expression levels without gene amplification is very unusual among various tumor cells. High expression levels of a single-copy N-*myc* gene have been reported to be typical for Wilms tumors [55,56]. Recently we found high N-*myc* expression levels in a human testicular tumor, without amplification of the N-*myc* gene [57]. Some SCLC cell lines express moderate levels of L-*myc* mRNA without the corresponding gene amplification [58].

ONCOGENIC POTENTIAL OF THE *MYC* GENES

Phenotypic Changes Induced by Activated *myc* Genes

The ability of the *myc* genes to produce a malignant phenotype has been directly shown in several cell culture models and more recently also in transgenic animals. The v-*myc* gene of the acutely transforming retroviruses causes a broad spectrum of avian malignancies and transforms fibroblasts and macrophages in vitro [59]. No apparent differences have been found in the ability of the v-*myc* gene to cause phenotypic changes in the target cells when compared with the c-*myc* gene under similar experimental conditions [60,61], suggesting that the oncogenic activation of the transduced v-*myc* gene in the avian retroviruses is based on its high level of expression driven by strong viral promoters. However, more recent studies have indicated that the v-*myc* gene has a higher transforming potential, suggesting that point mutations in viral *myc* genes might synergistically contribute to the oncogenic potential of the v-*myc* gene [62,63].

Highly expressed c-*myc* and v-*myc* gene constructs have been shown to reduce the requirements of established fibroblast cell lines for growth factors such as platelet-derived growth factor (PDGF) [60,61,64–66] and to abrogate interleukin-3 and interleukin-2 dependence of hematopoietic/lymphoid cells [67]. Recently it was shown that an N-*myc* expression vector transfected into fibroblasts can also induce cell division under growth factor-deprived conditions [68]. Fibroblast cell lines transfected with a c-*myc* gene have been found to be able to grow in semisolid medium and to be tumorigenic when inoculated into nude mice [66,69]. More recent findings have indicated that the N-*myc* gene can also cause complete transformation of at least one established fibroblast cell line (Rat-1A), as determined by morphology, soft-agar cloning, and tumorigenicity in nude mice [70].

On the other hand, primary embryonic fibroblasts transfected with a highly expressed c-*myc* or N-*myc* gene, or infected in vitro by v-*myc*-containing viruses, have

been shown to become "immortalized," i.e., to acquire an ability to grow continuously in cell culture, but were found to be nontumorigenic in vivo [60,71,72]. By contrast, the c-*myc*, N-*myc*, and L-*myc* genes have all been shown to be capable of fully transforming primary embryo fibroblasts when transfected together with an activated c-*ras* oncogene, which alone leads only to an immortalized but nontumorigenic phenotype [19,24,73,74]. An interesting example of other factors cooperating with a *myc* gene in transformation of normal cells was the finding that human B cells can be fully transformed when infected by an Epstein-Barr virus and subsequently transfected with the c-*myc* gene [75]. Possibly this mimicks the natural pathogenesis of Burkitt lymphoma.

myc Genes in Transgenic Mice

Direct experimental evidence of the oncogenic potential of the *myc* genes has been obtained by introducing *myc* gene constructs into transgenic mice. Mice carrying and expressing c-*myc* transgene driven by the mouse mammary tumor virus (MMTV) promoter were found to have a hereditary predisposition for the development of mammary carcinomas and occasional lymphomas [76]. Interestingly, when these mice were crossed with mice expressing an MMTV-v-Ha-*ras* transgene, a dramatic synergism was observed in the incidence of tumor formation [77]. However, the tumors arose stochastically and were monoclonal in origin, suggesting that additional somatic events were necessary for the pathogenesis of these tumors, even in the presence of activated c-*myc* and v-Ha-*ras* transgenes. Studies using MMTV-c-*myc* gene constructs that were anomalously expressed in a variety of tissues have suggested that the oncogenic potential of deregulated c-*myc* expression may vary in different tissues. An increased frequency of breast, testicular, lymphocytic (B- and T-cell), and mast cell tumors was observed, although many other tissues appeared to be unaffected despite a high expression of the MMTV-c-*myc* transgene [78].

The role of c-*myc* gene in naturally occurring leukemic disorders [45] is reflected also by studies on transgenic mice carrying immunoglobulin enhancer-driven *myc* gene constructs. The c-*myc* gene coupled to immunoglobulin μ or κ enhancers resulted in a high frequency of fatal lymphomas within a few months of birth, displaying several stages of B-cell maturation [79,80]. More recently, similar contructs containing the N-*myc* gene have also been shown to cause B-cell malignancies in transgenic mice [81,82].

REGULATION OF *MYC* GENE EXPRESSION

The c-, N-, and L-*myc* mRNAs are found in many tissues of the developing embryo, with distinct spatial and temporal patterns of expression, whereas in adult tissues expression of the c-*myc* gene predominates. The c-*myc* gene has been found to be expressed in all embryonic tissues studied [83–85]. By contrast, significant levels of N-*myc* mRNA have been found in only embryonic brain, kidney, intestine, lung, and heart and L-*myc* expression in brain, kidney, and lung of the embryo [56,84–87]. As a rough generalization from these studies, N-*myc* and L-*myc* expression might therefore be more tightly coupled to certain cell- and tissue-differentiation pathways, whereas c-*myc* expression seems to be mainly associated with cellular proliferation.

In normal adult tissues, the c-*myc* mRNA levels closely correspond to the proliferative activity of the corresponding tissues, with the exception of the actively

proliferating germinal epithelium of the testis, where c-*myc* expression can barely be detected [88]. By contrast, N-*myc* or L-*myc* mRNAs are present only in some adult tissues. A low level of N-*myc* expression has been found in adult testis, kidney, brain, heart, and pre-B cells [84,86]. In adult tissues, L-*myc* mRNA has so far only been detected in the lung [84], but the specific cell type responsible for this expression is not yet known. Recently we found that low-level L-*myc* expression takes place also in normal adult testicular tissue [89]. A differentiation-specific differential control of the *myc* genes has also been shown to exist in the adult organism; both c-*myc* and N-*myc* genes are expressed in normal pre-B cells, but at the later stages of B-cell differentiation the expression of the N-*myc* gene is shut off, and only c-*myc* RNA can be found [84].

Growth Factors Stimulate c-*myc* Expression

When quiescent cells are stimulated to divide by supplying their culture medium with growth factors, or various cell type-specific mitogens, the level of c-*myc* expression is rapidly increased [90–95]. Furthermore, c-*myc* expression has been shown to be induced in vivo in the regenerating liver of partially hepatectomized mice [96]. The activation of the c-*myc* gene in response to different growth-inducing stimuli appears to be mediated by diverse second messenger pathways. For example, the induction of c-*myc* by PDGF has been shown to be mediated mainly by the phosphoinositide-protein kinase C (PK-C)-dependent pathway, whereas the c-*myc* induction in response to the epidermal growth factor (EGF) is not associated with the activation of the PK-C second messenger system [92,93]. However, stimulation of cells with serum or purified growth factors results in complex changes in cellular biochemistry and gene expression [see, e.g., 97], and it is evident that neither the activation of PK-C nor the induction of c-*myc* expression alone is sufficient to explain the mitogenic effects of PDGF [64,93].

c-*myc* Expression During the Cell Cycle

Cell-cycle studies have indicated that the growth factor-stimulated induction of the c-*myc* gene is associated with the entry of resting cells into the cell cycle (see references cited above). The c-*myc* protein has been shown to act as a "competence" factor when microinjected into quiescent fibroblasts [98]. Transfection of murine erythroleukemia cells with an antisense c-*myc* vector has been shown to inhibit their G_1 progression [99]. A study on the effects of c-*myc* antisense oligonucleotides on human T-lymphocyte activation indicated, however, an inhibition of entry into the S phase [100]. In contrast to the mitogenic activation of c-*myc* in resting cells, the levels of c-*myc* mRNA in continuously cycling cells are invariant throughout the cell cycle [101]. Similarly, no cell cycle-dependent variation has been observed in the rate of synthesis or the total amount of the c-*myc* protein in continuously growing cells [102,103]. This does not, of course, rule out the possibility of cell cycle-specific modifications of the c-*myc* protein.

Down-Regulation of c-*myc* Expression

A marked down-regulation of c-*myc* expression has been found to be an early response of cultured cells to interferon treatment [104,105], suggesting that attenuation of c-*myc* activity could have a role in the antiproliferative action of interferons. It has been proposed that interferons might represent a physiological feedback mechanism for down-regulation of growth factor-activated genes, such as the c-*myc* gene [106–108].

Experimental inhibition of c-*myc* expression by different antisense constructs has been shown to inhibit cell growth and induce the differentiation of several types of cells [99,100,109–111]. Also, down-regulation of c-*myc* mRNA has been implicated in several in vitro differentiation models as a response to treatment of the cells with various differentiation-promoting agents, such as retinoic acid (RA) [112–115]. The receptor mediating the effects of RA is a member of the steroid receptor family in the nucleus [116]. However, no interactions between the RA–receptor complex and the regulatory sequences of the *myc* genes have been established.

Modulation of N-*myc* and L-*myc* Expression

The cellular regulation of the activities of the N-*myc* and L-*myc* genes appears to be very different from that of the c-*myc* gene, and attempts to modulate N-*myc* or L-*myc* expression experimentally have so far been a less rewarding area of research. No findings have been reported on significant activation of N-*myc* or L-*myc* genes in response to peptide hormones or other inducers of cell proliferation. This may be due in part to a lack of suitable experimental models, since the tumor cell lines expressing N-*myc* or L-*myc* mRNA often also have an amplification of the corresponding gene, which may make them unresponsive to physiological N-*myc* or L-*myc*-inducing stimuli, respectively.

N-*myc* mRNA decreases during the differentiation of human neuroblastoma cells induced with RA or phorbol ester [117,118]. c-*myc*, N-*myc*, and L-*myc* mRNAs have all been found to be expressed in murine embryonal carcinoma (EC) cells and to be down-regulated upon their RA-induced endodermal differentiation [119–121]. However, studies by Finklestein and Weinberg [120] have shown that the repression of N-*myc* mRNA expression is only transient and then returns to initial levels at the later stages of endodermal differentiation.

Molecular Mechanisms of *myc* Gene Regulation

The accumulation of c-*myc* mRNA in growth-arrested murine fibroblasts in response to serum stimulation is due to an increased initiation of transcription [122,123] and, in particular, to a concomitant rapid stabilization of c-*myc* mRNA [123,124]. By contrast, these mechanisms do not significantly contribute to the c-*myc* induction in the same cells in response to EGF, which was found to be caused by a release of intragenic pausing of c-*myc* transcription [123]. Analogously, increased c-*myc* expression in T cells activated by the calcium-channel modulator ionomycin has been attributed to an increased transcriptional initiation, but the PK-C-activating phorbol ester TPA was shown to increase c-*myc* RNA in T cells by removal of an intragenic block of transcript elongation [125].

A block of transcriptional elongation down-regulates the expression of the amplified c-*myc* gene during dimethylsulphoxide-induced granulocytic differentiation of HL-60 leukemia cells [126,127]. However, during a later stage of the differentiation of the HL-60 cells, a decrease of transcriptional initiation was observed [127]. On the other hand, negative posttranscriptional controls, i.e., mRNA destabilization, have been indicated in the down-regulation of the c-*myc* [123,128] and N-*myc* [119] expression during the differentiation of the F9 embryonal carcinoma cells into visceral endoderm-like cells.

Several DNAse I hypersensitive sites have been identified in the c-*myc* gene [129–131]. They are believed to be sites of interaction of chromatin and nonhistone

proteins and thus represent potentially important regulatory regions. Protein occupancy of some of these sites is correlated with the transcriptional status; some of them are associated with known regulatory elements of the c-*myc* gene [126,127].

Cis-Acting Sequences in the Regulation of the *myc* Genes

The activity of the c-*myc* promoter region is controlled by a cooperation of positively and negatively acting factors. Several DNA regions mediating this regulation (cis-acting elements) have been identified in the c-*myc* gene. Transfection experiments using different regulatory sequences from the 5′ region of the murine c-*myc* gene linked to a reporter gene have identified a negative cis-acting element located 400–1,200 bp upsteam of the P1 promoter [132] and a positively acting element encompassing approximately 400 bp downstream of c-*myc* promoters P1 and P2 in the first intron of the gene [133]. Both negatively and positively regulating cis-acting regions have also been demonstrated in the human c-*myc* gene [50,134–137].

Chung et al. [134] and Lipp et al. [135] have examined the activity of c-*myc*-reporter gene constructs transfected together with short competing DNA sequences from the corresponding regulatory regions of the c-*myc* gene. However, the results of these studies show considerable variation, which might indicate cell type-specific differencies in the molecular mechanisms controlling c-*myc* expression. Interestingly, recent findings by Hay et al. [136] show that a transcription factor complex including the oncoproteins encoded by the c-*fos* and c-*jun* genes binds to the 5′-regulatory sequences of the c-*myc* gene. The sequence mediating this binding resides in a region previously identified as a negatively acting regulatory element [137], but the significance of this interaction in the normal regulation of the c-*myc* gene remains to be demonstrated.

Attempts to define cis-acting regulatory sequences in the N-*myc* gene have not been very successful. No differences in the activity of the transfected reporter gene have been observed when linked with different portions of the putative N-*myc* regulatory sequences. Also, the activity of N-*myc*-reporter gene constructs and the activity of an intact N-*myc* gene have been similar with introduction into cells actively expressing an endogenous N-*myc* gene or into cells in which the N-*myc* gene is normally silent [138]. However, an introduced intact N-*myc* gene containing the corresponding regulatory sequences was found to be under normal tissue-specific and developmental control in transgenic mice [81].

Posttranscriptional Regulation of *myc* Gene Expression

The rapid turnover of c-*myc* mRNA [139] makes its expression a feasible target of positive and negative regulation by modulation of its stability and thus its half-life in cells. Sequences responsible for the instability of c-*myc* mRNA have been mapped in the untranslated regions of exon I and the 3′ end of exon III [140,141]. Analysis of c-*myc* deletion mutants in stably transfected fibroblast cell lines located the sequences primarily responsible for the short c-*myc* mRNA half-life to a region of 140 bases, in the 3′ untranslated region [140]. This AT-rich region contains three copies of the AUUUA motif, which has been identified in a variety of mRNAs with a short half-life and has been shown to mediate a rapid mRNA degradation when introduced into the otherwise stable β-globin mRNA [142].

These studies also indicate a role, albeit less pronounced, for c-*myc* exon I in the mRNA instability [140]. This is consistent with the data on naturally occurring

mutations of the c-*myc* gene in tumor cells. The c-*myc* mRNA has been found to be three to ten times more stable in tumors in which the first exon of the c-*myc* gene has been truncated or deleted [46,143]. The sequences transcribed from exon I have been found to be important for the rapid turnover of the c-*myc* mRNA also in a cell-free in vitro study system [141]. However, these sequences were not able to mediate rapid RNA degradation when tagged to heterologous mRNAs, suggesting an interaction with other c-*myc* mRNA structures or with the translated c-*myc* protein. An early step in the c-*myc* mRNA degradation is the shortening of the poly-(A) tail and the degradation of the 3′ AU-rich sequences [144]. In many cell types, however, a more stable, nonpolyadenylated c-*myc* mRNA population can be identified [145].

Since inhibition of protein synthesis is known to induce c-*myc* expression and to potentiate the accumulation of c-*myc* mRNA in response to serum, mainly because of c-*myc* mRNA stabilization [90,997,146], a role for short-lived proteins in the degradation of c-*myc* mRNA has been postulated. However, the very immediate nature of c-*myc* induction in cells exposed to protein synthesis inhibitors does not favor such a hypothesis. A more likely possibility is that a halt in the translation of the c-*myc* mRNA per se causes its stabilization. Ongoing translation has been shown to be required for the proper degradation of the mRNAs of β-tubulin [147] and c-*fos* [148] genes. Interestingly, in the former case mRNA instability was found to be cotranslationally autoregulated by the aminoterminal residues of the nascent β-tubulin polypeptide chain.

The turnover of N-*myc* mRNA is not as rapid as that of c-*myc* mRNA. The N-*myc* mRNA half-life in neuroblastoma cells, which have an amplified and abundantly expressed N-*myc* gene, is about 40 min (T.P. Mäkelä, unpublished results) compared with 10–20 min reported for c-*myc* mRNA [139]. However, inhibition of protein synthesis leads to the stabilization of N-*myc* mRNA as well (T.P. Mäkelä, unpublished results).

Regulation of the Transcriptional Elongation of the *myc* RNAs

Nuclear run-off experiments have indicated that regulation of transcriptional elongation across the exon 1–intron 1 boundary is a critical determinant of cellular c-*myc* mRNA levels [126]. More recent studies on *Xenopus* oocytes injected with plasmids containing c-*myc* sequences [149] and studies on in vitro c-*myc* transcription using purified RNA polymerases II and III [150], have located sequences specifying this premature transcriptional termination to a 95 bp region in the 5′ end of the first exon of the human c-*myc* gene. It has been proposed that the transcriptional initiation from the superimposed promoters for the RNA polymerases II and III in the c-*myc* gene might serve an important function in the regulation of the elongation of the c-*myc* transcripts [151]. The termination activity of these sequences was found to be orientation-specific and was demonstrated to program transcriptional termination when introduced downstream of a heterologous promoter [149].

The factors binding to these sequences have not yet been characterized. This block of elongation can be partially overcome by inhibition of protein synthesis [152], suggesting the involvement of labile regulatory proteins. However, both treatment of cells with an RNA-intercalating agent proflavin and treatment of isolated nuclei with RNaseA prior to the run-off experiments have also been reported to allow transcription to proceed into regions downstream of the exon 1–intron 1 boundary of the c-*myc* gene [153,154].

The studies on small-cell lung cancer (SCLC) cell lines expressing elevated levels of different *myc* mRNAs have indicated a role for premature transcriptional termination in the regulation of steady-state levels of c-*myc* and L-*myc* but not N-*myc* mRNA [58]. High levels of L-*myc* mRNA in a cell line with a single-copy L-*myc* gene were associated with reduced intragenic transcriptional pausing between exon 1 and exon 2 compared with the cell lines having an amplified gene [58].

Antisense Transcription

A surprising finding in the study of the transcriptional regulation of the c-*myc* gene was that transcription also occurs in the antisense strand [152]. This is especially charasteristic of the murine c-*myc* gene, whereas antisense transcription of the human c-*myc* gene usually takes place only in the sequences upstream of the P1 promoter [28,50]. The antisense transcription of the murine c-*myc* gene is constitutive, and it is not coregulated with the transcription of the sense strand, e.g., in response to serum stimulation [152]. Antisense transcription has also been found in the actively sense-transcribed human N-*myc* gene [58]. Despite the existence of antisense c-*myc* transcription, no distinct c-*myc* antisense RNAs have so far been identified in any cells [152,154]. Altogether, the physiological significance of the *myc* antisense transcription, if any, remains to be demonstrated.

Turnover and Structure of L-*myc* mRNA

We have used the SCLC cell line U-1690, having an amplified and abundantly expressed L-*myc,* gene as a model system to study turnover and regulation of L-*myc* mRNA [44]. This was found to differ in several ways from what is known about the c-*myc* and N-*myc* genes. However, some kind of cross regulation between the different *myc* genes seems to exist; the U-1690 cells, in contrast to other SCLC lines and tumor cell lines in general, failed to express any detectable c-*myc* mRNA. Similar findings have been reported by others [54,55].

Northern blotting analysis of L-*myc* mRNA from U-1690 cells revealed an intense 3.8 kb band and a fainter 2.2 kb band hybridizing with the L-*myc* probe [44]. These two different L-*myc* mRNA classes are produced by alternative RNA processing [23,43]. The smaller 2.2 kb mRNA lacks the third exon L-*myc* sequences and terminates in a consensus polyadenylation sequence in the second intron of the L-*myc* gene. Using the RNAse protection assay, we found further heterogeneity in the 3.8 kb and 2.2 kb L-*myc* mRNAs, already suggested by their diffuse appearance in Northern blotting analysis. Hybridization with a radioactive L-*myc* antisense RNA probe and subsequent digestion of unhybridized single-stranded RNA resulted in two distinct protected fragments, indicating differential splicing of the first intron of the L-*myc* gene, as described by Minna and collaborators [23,43]. When examining L-*myc* RNA from normal tissues using RNAse protection assay, we found the corresponding two fragments protected by RNA prepared from testicular tissue. Thus L-*myc* mRNA is present in adult testicular tissue and is also differentially spliced in normal cells.

The turnover of L-*myc* mRNA was studied using actinomycin D, an inhibitor of RNA synthesis. Inhibition of transcription in U-1690 cells resulted in a relatively rapid decrease of the 3.8 kb L-*myc* mRNA, whereas the 2.2 kb form was more stable. Thus it was concluded that the two L-*myc* mRNA classes have different half-lives, suggesting that some sequences present in the 3.8 kb RNA, but not in the 2.2 kb RNA, are

responsible for its more rapid degradation in U-1690 cells. The half-life of the 3.8 kb RNA was approximately 45 min, whereas the half-life of the 2.2 kb RNA was about 2 h. The relatively rapid turnover of L-*myc* mRNA suggested that, like c-*myc* and N-*myc* mRNA, it might also be stabilized by inhibition of protein synthesis. To test this hypothesis, the half-life of L-*myc* mRNA was measured in the prescence of cycloheximide, a potent inhibitor of protein synthesis. These studies indicated that the half-life of L-*myc* mRNA was not affected by the translational arrest, suggesting that posttranscriptional mechanisms are not involved in the regulation of L-*myc* mRNA expression in U-1690 cells. However, cell type-dependent differences in the regulation of L-*myc* mRNA may exist; our recent data indicate that stabilization of L-*myc* mRNA following exposure to protein synthesis inhibitors occurs in certain L-*myc*-expressing cell lines. Furthermore, a low level of L-*myc* mRNA detected in an embryonal stem cell line was recently shown to be slightly increased following inhibition of protein synthesis [121], suggesting that stabilization of the L-*myc* mRNA might be involved in the regulation of L-*myc* mRNA expression in some cells.

Strikingly, kinetic analyses of L-*myc* mRNA levels in U-1690 cells treated with cycloheximide alone indicated that L-*myc* mRNA was down-regulated by the inhibition of protein synthesis [44]. Since the half-life of L-*myc* mRNA was found to be constant during the experiment, it is concluded that the decline of L-*myc* mRNA was due to decreased transcription of the L-*myc* gene. After a lag period of about 45 min, the cellular levels of the major 3.8 kb L-*myc* RNA began to decrease, with a similar decay rate as was seen in cells in which transcription was inhibited. This kinetic pattern strongly suggested that the expression of the L-*myc* gene in U-1690 cells is dependent on positively acting labile proteins and is down-regulated when the cellular levels of these regulatory proteins fall under a critical threshold level. It may well be that depletion of such proteins becomes a rate-limiting step, particularly in cells that have an amplified number of L-*myc* gene copies competing for the same trans-acting factors. A similar protein synthesis-dependent transcriptional regulation has been shown to take place in bursal lymphoma cells, in which the c-*myc* transcription is regulated by avian leukosis virus (ALV) long terminal repeat (LTR) sequences, whereas the transcription of a normally regulated c-*myc* gene was not affected, or was slightly increased, following inhibition of protein synthesis [146]. The novel techniques for studying DNA–protein interactions should allow us to characterize such putative proteins and their binding sites in the regulatory regions of the L-*myc* gene.

PROTEINS ENCODED BY THE *MYC* GENES

Characterization of the *myc* Proteins

The v-*myc*-encoded protein was first identified as a 110 kd gag–*myc* fusion protein from avian cells transformed by the MC29 virus [155,156]. The production of *myc*-specific polyclonal and monoclonal antibodies confirmed the nuclear localization of the v-*myc*-encoded proteins and allowed the characterization of the nuclear, short-lived, DNA-binding phosphoprotein encoded by the c-*myc* gene [18,157–162]. Phosphorylation, rapid turnover, and nuclear localization have thereafter been shown also to characterize the proteins encoded by the N-*myc* [163–165] and L-*myc* genes [166,167]. The exceptionally short half-life of the c-*myc* protein ($\approx$20 min in most cells) has been shown to be controlled by a degradation mechanism requiring metabolic energy and has been found to be increased in heat-shocked cells [168]. Recently it was reported that

casein kinase II can phosphorylate c-*myc* and v-*myc* proteins in vitro [169]. The in vitro phosphorylated *myc* proteins generated proteolytic phosphopeptides corresponding to those of the *myc* proteins from avian and human cells, suggesting a functional role for casein kinase II in phosphorylation of the v-*myc* and c-*myc* proteins in vivo.

Besides phosphorylation, no other posttranslational modifications of the c-*myc*, N-*myc*, or L-*myc* proteins have been reported. The apparent molecular weights of the *myc* proteins in polyacrylamide gel electrophoresis are considerably larger than deduced from their amino acid sequences. For example, the predicted molecular weight of the 62 kd c-*myc* polypeptide is only 49 kd. It is unlikely, however, that this discrepancy can be explained on the basis of yet unknown posttranslational modifications, since the electrophoretic mobility of c-*myc* protein produced from a full-length human c-*myc* cDNA in *Eschrichia coli* cells was found to identical to that of the c-*myc* protein immunoprecipitated from Burkitt lymphoma cells [18].

Subnuclear Localization of the c-*myc* Protein

With immunofluorescence microscopy, the nuclear distribution of v-*myc* and c-*myc* proteins appears as a speckled pattern occupying a portion of the nucleoplasm, excluding the nucleoli and nuclear pore–lamina complex [158,170,171]. Interestingly, despite the ability of *myc* proteins to bind DNA in vitro, our immunofluorescence studies on the distribution of the v-*myc* protein in MC29-infected cells during mitosis showed no association between condensed chromatin and the *myc* proteins [170].

Early studies on the subnuclear localization of the *myc* proteins indicated an association with the nuclear matrix [172]. These results were challenged by Evan and Hancock [173], who showed that the c-*myc* protein is normally soluble and that its binding to the nuclear matrix may represent an artefact of nuclear fractionation procedures. Association with nuclear matrix components was shown to be induced by a heat shock and also by exposing isolated nuclei to physiological temperatures. More recently it was found that c-*myc* protein associates with the nuclear matrix after treatment of cells with $CuSO_4$. The c-*myc* protein thus associates with the nuclear fraction known as the Cu^{2+}-induced nuclear matrix I [174].

Functional Domains of the c-*myc* Protein

The regions of extensive amino acid sequence homology between the different *myc* proteins are confined to distinct regions, which are thought to represent functional domains critical for those cellular functions shared by all *myc* proteins. Accordingly, although there are regions of difference between the different *myc* proteins, there is a high degree of homology between the corresponding *myc* proteins of different species. There may thus be important functions unique to the individual members of the *myc* gene family.

In vitro mutagenesis experiments with the c-*myc* gene have indicated that some of the conserved regions can be deleted from the c-*myc* protein without affecting the ability of the mutated c-*myc* gene to collaborate with the c-Ha-*ras* oncogene in transformation of primary rat embryo fibroblasts [70,175]. These studies suggested that amino-terminal regions encoded by the second exon and carboxy-terminal regions encoded by the third exon of the c-*myc* gene are particularly critical for the oncogenic action of the c-*myc* protein. In the study by Small et al. [70] the ability of the mutated *myc* genes to transform established fibroblasts (a Rat-1-derived cell line) without the assistance of

other oncogenes was also evaluated. Interestingly, distinct regions were found that could be uncoupled from the cotransforming activity of the c-*myc* gene but were indispensable for the transformation of the Rat-1 cells caused by an introduced c-*myc* gene alone and vice versa. However, it is clear that mapping of regions causing loss of function is insufficient for the assignment of specific functional domains of a protein, since deletions can have unpredictable effects on the higher-order structure, stability, or cellular localization of the mutated proteins.

Based on sucrose gradient fractionation of the $p110^{gag\text{-}myc}$ protein from MC29 infected quail cells, Bader and Ray [176] suggested that *myc* proteins might appear as dimers. Interestingly, the recent results of Dang et al. [177] have shown via gel filtration chromatography that purified recombinant human 62 kd c-*myc* polypeptides occur as M_r 150,000–250,000 oligomers after in vitro glutaraldehyde cross linking, whereas mutant c-*myc* polypeptides deleted of the carboxy-terminal residues 410–439 containing a periodic array of leucine residues, termed the leucine zipper [178], occurred only as monomers. Since this same region has been found to be critical also for cellular transformation by the c-*myc* gene [70], it is suggested that oligomerization mediated by a distinct secondary structure in this region might be necessary for the oncogenic action of the c-*myc* protein. However, using a reversible cross-linking agent, dimethyl 3,3′-dithiobis-propionimidate, no *myc* protein oligomerization was observed in v-*myc* expressing MH2 cells by Gillespie and Eisenman [179]. Instead, they found that a single $M_r \approx$ 500,000 nuclear protein designated MYAP (*myc*-associated protein) could be specifically cross linked with the v-*myc* protein in these cells. In remains to be determined whether this protein is associated with *myc* protein via a specific leucine zipper interaction.

Nuclear Transport Signal of the c-*myc* Protein

Two specific regions responsible for the nuclear localization of the c-*myc* protein have been characterized in its carboxy-terminal one-third [180,181]. A peptide of nine amino acids (residues 320–328, counting from the "classic" ATG initiation codon giving rise to a predicted 439 aa protein) was identified as the major nuclear transport signal of the c-*myc* protein. In addition, amino acid residues 364–374 were found to be able to provide an incomplete nuclear localization for mutant c-*myc* proteins deleted of the major nuclear transport signal. A synthetic peptide corresponding to the major nuclear transport signal of the c-*myc* protein was found to target efficiently nuclear localization when conjugated to human serum albumin and microinjected into the cytoplasm cells, whereas a peptide corresponding to the latter amino acid sequence caused a slower and only a partial nuclear redistribution [181].

Peptide sequences reminiscent of the c-*myc* nuclear targeting signal, though not identical, have been shown to be responsible for the nuclear localization of polyomavirus and Simian virus 40 T antigens [182,183]. Similar sequences can also be identified in the predicted human N-*myc* protein, but not in the L-*myc* protein. However, the sequences encoding the major nuclear transport signal could be deleted from the c-*myc* gene without affecting its activity in the cotransformation assay. By contrast, residues 364–374, also associated with the nuclear location of the c-*myc* protein, are highly conserved in the predicted N-*myc* and L-*myc* proteins and were found to be critical for the oncogenic potential of the c-*myc* protein [181].

Polypeptide Heterogeneity of the c-*myc* Protein

The human c-*myc* protein and c-*myc* proteins from other species appear as polypeptide doublets in gel electrophoresis (apperent molecular weights 62,000 and 64,000) [13,159,160,184]. It has not been possible to explain this heterogeneity by posttranslational modifications, and its molecular basis has remained unknown until recently. Studies by Hann et al. [51] showed that the translation of the higher molecular weight form of the c-*myc* protein (M_r 64,000) is initiated from a CUG codon in the first exon of the c-*myc* gene, previously considered to be completely noncoding.

Interestingly, the synthesis of the higher M_r c-*myc* polypeptide is disrupted due to removal or mutations of the first exon of the c-*myc* gene in many Burkitt lymphoma cell lines [51,184], potentially contributing to the oncogenic activation of c-*myc* in Burkitt lymphoma. Further experimentation is clearly needed to establish whether the normal and aminoterminally truncated c-*myc* proteins have important functional differences and to test the intriguing possibility that the two c-*myc* polypeptides could modulate their action, e.g., by interacting with each other or by competing for common cellular target molecules.

A Provocative Open Reading Frame (ORF) in the First Exon of the Human c-*myc* Gene

Examination of the published sequence of the human c-*myc* gene has revealed a provocative short ORF in the first exon of the gene, having a potential coding capacity for a polypeptide of 188 residues [185]. However, the significance of this observation is unclear, since such an ORF is not present in the genomic sequences of the c-*myc* genes of any other species determined so far, and it is a matter of controversy whether proteins encoded by the human c-*myc* first exon ORF do exist in any cells. Gazin et al. [186] reported that a 32 kd protein and its (apparently) dimeric 58 kd form is recognized in several human cell lines by polyclonal antisera produced against various peptides corresponding to different, nonoverlapping parts of the first exon ORF. These findings were contradicted by Ferfe et al. [187], who failed to identify any exon 1-related proteins in human cells using an antiserum that detects such proteins in cells tranfected with a corresponding recombinant construct. Also, it could be argued that the amounts of the 58 kd and 32 kd proteins detected by Gazin et al. [186] do not appear to correlate very well with the c-*myc* mRNA levels present in the corresponding cell lines.

N-*myc* Protein

The putative amino acid sequence of the N-*myc* protein product was first derived from the long ORF located in the second and third exons of the N-*myc* gene [20,188]. Soon thereafter, the N-*myc* protein was identified as an approximately 66 kd polypeptide doublet in sodium dodecyl sulfate (SDS) electrophoresis [163–165].

We have studied the N-*myc* protein using a polyclonal antiserum produced against a bacterially expressed fragment from the second exon, close to the N-terminus of the N-*myc* protein [189]. Immunoprecipitations with this antiserum from several cell lines expressing amplified N-*myc* genes revealed considerable heterogeneity of the N-*myc* polypeptides with four closely migrating bands at 58–64 kd. This heterogeneity could be in part attributed to posttranslational modification by protein phosphorylation; treatment of the immunoprecipitates with alkaline phosphatase converted the four bands into the two faster migrating bands (p58 and p60). This "band shift" seen in methionine-

labeled N-*myc* protein correlated with total loss of phosphate label from the immunoprecipitated protein and could be specifically blocked with *para*-nitro-phenyl-phosphate, demonstrating that it is indeed a dephosphorylation event. A similar but even more pronounced "band shift" was observed in phosphatase-treated L-*myc* but not c-*myc* immunoprecipitates [166].

Curiously, one of our N-*myc* deletion mutant constructs transiently expressed in monkey COS cells produced a polypeptide doublet that does not show a mobility shift in SDS gels when dephosphorylated but is as efficiently metabolically labeled with radioactive phosphorus as is the wild-type form of N-*myc* protein. This together with our unpublished data on N-*myc* and L-*myc* proteins and data presented by Saksela et al. [166] suggest that the mobility shift of the N-*myc* and L-*myc* proteins upon phosphorylation cannot be explained solely on the basis of the increased net incorporation of phosphate. We have tentatively mapped the region critical for this "band shift" to a part of the second exon of both the N-*myc* and the L-*myc* genes, and we are currently further characterizing this region by mutagenesis. Interestingly, several other nuclear phosphoproteins, including the c-*fos* [190], c-*ets*-1, c-*ets*-2 [191], and *Rb* gene products [192] and the *myo*DI protein (H. Weintraub, personal communication) show a similar mobility shift upon phosphorylation.

Even after dephosphorylation, the immunoprecipitated N-*myc* protein appeared as two bands of similar relative amounts in all cells studied, and the two bands showed no apparent precursor–product relationship in a pulse-chase experiment. Furthermore, a genomic expression vector containing all three exons of the N-*myc* gene produced a similar pattern of two bands in COS cells. Using deletion constructs of the expression vector, we were able to locate the origin of the two bands close to the N-terminal end of the protein, and in vitro mutagenesis of the N terminus confirmed that the difference in the two N-*myc* protein bands is in their primary structure [189]. The N-*myc* protein is translated from two alternative AUG initiation codons, which are located 24 nucleotides apart; thus the N-*myc* protein consists of two separate polypeptides differing by an N-terminal stretch of eight amino acids, termed N-*myc*-1 (465 aa) and N-*myc*-2 (458 aa). Here again the N-*myc* protein shows a similarity with the c-*myc* protein, which also has been found to consist of two separate polypeptides due to differential translational initiation [51] (see above). The physiological significance of the two distinct N-*myc* and c-*myc* polypeptides is unclear; the eight amino acid "overhang" of N-*myc* does not show homology to the corresponding 15 amino acid overhang of c-*myc* nor to other protein sequences available. However, the presence of two translational initiation codons in both N-*myc* and c-*myc* of mouse and man [51] (T. Mäkelä, unpublished results) suggests a functional significance for this phenomenon.

L-*myc* Protein

The differential splicing and use of multiple polyadenylation sites by L-*myc* mRNA synthesis provide several possible ORFs with potential capacities to encode polypeptides with very different primary structures [23]. The first means of detecting proteins encoded by the L-*myc* gene was provided by the production of polyclonal and monoclonal antibodies raised against a synthetic peptide corresponding to a region homologous in the predicted proteins of all the sequenced cellular and viral *myc* genes (pan-*myc* antibodies [166,193]). Comigrating proteins have also been identified by

SDS-PAGE with an antiserum raised against a bacterially produced L-*myc* antigen [167].

The SCLC cell lines U-1690 and COR L88 express high levels of L-*myc* mRNA, but not c-*myc* or N-*myc* mRNA, and thus provided a suitable study system for the characterization of the L-*myc* protein using pan-*myc* antibodies. Analysis of the immunoprecipitates in SDS-PAGE revealed a triplet of M_r 60,000–66,000 polypeptide bands from these cell lines but not from cells devoid of L-*myc* expression [166]. These polypeptides could be precipitated with rabbit and sheep polyclonal and mouse monoclonal pan-*myc* antibodies but not with normal rabbit serum. Furthermore, this immunoprecipitation could be abolished by prior incubation of the pan-*myc* antibodies with the corresponding peptide immunogen or with bacterially produced recombinant L-*myc*-β-galactosidase fusion protein, strongly suggesting that the M_r 60,000, 64,000, and 66,000 proteins are encoded by the L-*myc* gene. A corresponding triplet of proteins was observed in autoradiography when the L-*myc*-expressing cells were labeled either with radioactive methionine or phosphate prior to the immunoprecipitation analysis, indicating that all L-*myc* polypeptide species are phosphorylated.

To study the nature of L-*myc* protein phosphorylation, the phosphoester bonds of L-*myc* polypeptides were subjected to enzymatic hydrolysis with calf intestinal alkaline phosphatase (CIP). We found that treatment of the L-*myc* immunoprecipitates with CIP prior to SDS-PAGE converted the three methionine-labeled polypeptides into a single band migrating at M_r 59,000 and efficiently removed radioactivity from the phosphate-labeled L-*myc* protein [166]. This indicated that, in contrast to the c-*myc* and N-*myc* proteins, the L-*myc* polypeptide heterogeneity is due solely to differential phosphorylation of a common precursor. While our report on L-*myc* protein phosphorylation was in press, De Greve et al. [167] published their studies on the L-*myc* protein. Based on in vitro translation of two distinct L-*myc* gene constructs, they concluded that the different forms of the L-*myc* protein arise from differential translational initiation, the 66 kd form of the L-*myc* protein being translated from a CUG codon in the first unspliced intron present in a subpopulation of L-*myc* mRNA. We have no explanation for the apparent discrepancies between their data and our data.

To confirm our conclusions on the polypeptide composition of the L-*myc* protein, we constructed an SV40 early promoter-driven expression vector coding for most of the L-*myc* second exon but excluding sequences from intron 1 or exon 1, thus including only one potential translational initiation site, the ATG codon in the 5′ end of the second exon of the L-*myc* gene. We found that a triplet of M_r 15,000–20,000 polypeptide bands is specifically precipitated from lysates of cells transfected with this expression vector. Furthermore, similarly to the wild-type forms of L-*myc* protein, these transiently expressed truncated L-*myc* proteins converted to a single polypeptide band when the L-*myc* immunoprecipitates were dephosphorylated prior to the SDS-PAGE analysis. These results substantiate our conclusion that the electrophoretic heterogeneity of the L-*myc* protein is due only to differential posttranslational phosphorylation and also indicate that the 137 amino-terminal amino acids of the L-*myc* protein are sufficient to produce this characteristic electrophoretic heterogeneity.

Interestingly, we found that the phosphopeptide pattern of the L-*myc* protein can be experimentally modulated. Addition of phorbol ester tumor promoter TPA to cultures of methionine-labeled L-*myc*-expressing cells caused a rapid and dramatic change in the electrophoretic mobility of the L-*myc* polypeptides [166]. In contrast to the steady-state

situation, TPA treatment resulted in the migration of most of the labeled L-*myc* protein, with the mobility of the highest apparent molecular weight L-*myc* polypeptide (M_r 66,000) by SDS-PAGE. A similar but less pronounced change in the proportion of the differentially phosphorylated forms of L-*myc* protein was also seen when the labeled cells were stimulated with fetal calf serum before immunoprecipitation analysis. In both cases, treatment of the samples with CIP again reduced the modified polypeptide bands to a single, fast-migrating polypeptide species.

The subsequent experiments indicated that a similar shift in the mobility of the L-*myc* polypeptides could also be obtained by treatment of the cells with 1-oleyl-2-acetylglycerol, a synthetic analogue of the physiological PK-C activator diacylglycerol, but not in response to nonactivating 4β-phorbol, strongly suggesting that this change in L-*myc* protein phosphorylation was mediated via PK-C. By contrast, addition of dibutyryl-cAMP or forskolin to cultures of L-*myc* expressing cells did not result in any change in the L-*myc* polypeptide pattern, suggesting that cAMP-dependent protein kinase(s) is not involved in this effect.

A role for phosphorylation of cytoplasmic protooncogene products in signal transduction and regulation of cell growth is well established [194–196]. Recent studies have shown that posttranslational modification can regulate the activity of certain nuclear transcription factors [197–199]. Interestingly, a number of nuclear oncoproteins, such as the c-*erb*A, c-*fos,* and c-*jun* proteins, have proved to function as transcription factors [200–204]. On the other hand, phosphorylation of the nuclear oncoproteins encoded by c-*fos,* c-*erb*A, and c-*ets*-1 genes has been found to be rapidly modulated by exogenous stimuli [190,191,200]. Thus, it appears likely that phosphorylation also plays an important role in the regulation of nuclear oncoprotein function.

In comparing the PK-C mediated phosphorylation of the L-*myc* protein with other recently published findings, it seems that phosphorylation of nuclear oncoproteins is regulated by multiple mechanisms. Whereas the phosphorylation of c-*erb*A protein was found to be stimulated by activators of PK-C as well as by activators of PK-A [200], neither of these kinases seemed to be involved in the TPA-induced phosphorylation of the c-*fos* protein [190] or in the calcium-dependent phosphorylation of c-*ets*-1 protein [191]. We do not know whether the phosphorylation of the L-*myc* protein in response to TPA is a direct effect of PK-C or is mediated via the multiple steps also involving other kinases. However, the immediate nature of this phenomenon and its independence of protein synthesis indicate that this PK-C-dependent pathway already exists in unstimulated cells. It should be noted also that recent investigations have revealed that PK-C, in fact, consists of a family of closely and also more distantly related TPA-activated kinases [205–207], which may differ in their modes of regulation as well as in their target specificity.

Consensus nucleotide motifs have been identified in the 5′ portions of several genes whose expression is enhanced by TPA [208–210]. The affinity of the transcription factor c-*jun*/AP-1 for these TPA-responsive DNA elements appears to be regulated by TPA-induced posttranslational modifications [208]. Since c-*fos* and c-*jun*/AP-1 proteins act together in mediating the transcriptional effects of TPA [204,211–213], it may be that the TPA-induced phosphorylation of the c-*fos* protein [190] has an important role in the regulation of this interaction. It is tempting to speculate that the as yet unknown functions of the L-*myc* protein also could be modulated by the PK-C-mediated phosphorylation reported earlier [166]. The unusually large shift in the electrophoretic

mobility of the L-*myc* protein makes it reasonable to suggest that these modifications alter the higher order structure and thereby other functionally relevant properties of this protein as well.

Interestingly, bombesin-like peptides, which have been shown to be growth factors for human bronchial epithelial cells and SCLC [214–217], are also known to be potent activators PK-C [218–220]. Transduction of biochemical signals from PK-C to the L-*myc* protein could therefore be physiologically significant in normal and malignant lung cells. Studies by Ramsay et al. [221] have shown that loss of the v-*myc* protein phosphorylation sites from mutant MC29-type virus strains greatly diminishes the oncogenic potential of these viruses, whereas back-mutants regain transforming properties similar to the wild-type viruses. Future transfection experiments with mutagenized L-*myc* gene constructs are needed to examine whether similar correlations exist in the case of L-*myc* protein phosphorylation.

On the other hand, it is also possible that the observed differential phosphorylation of the L-*myc* protein reflects some cell cycle-regulated changes in its properties or distribution, which may not be directly involved in cellular growth control. For example, the reversible depolymerization of the nuclear lamina during mitosis has been suggested to be regulated by differential phosphorylation of the major nuclear lamina proteins, the lamins [222]. Recently it was reported that lamin B is also rapidly phosphorylated after activation of PK-C [223]. Histones, the major structural proteins of chromatin, have been shown to become hyperphosphorylated in mitosis [224] as well as in response to TPA [225,226]. Also, the c-*myc* protein has been found to be hyperphosphorylated during mitosis (B. Lüscher and R.N. Eisenman, personal communication). This effect is mediated by a casein kinase II-independent pathway and results in a large shift in the electrophoretic mobility of the c-*myc* protein. We have previously found that the in vitro DNA-binding v-*myc* protein is not associated with chromatin in mitotic cells [170]. On the other hand, phosphorylation of the c-*fos* protein [227,228] and the SV40 T antigen [229] is inversely correlated with their DNA-binding activities in vitro. Thus, analogous to these studies, the PK-C-modulated differential phosphorylation of the L-*myc* protein might be associated with such changes in the nuclear biochemistry, e.g., during mitosis.

FUNCTIONS ATTRIBUTED TO THE *MYC* PROTEINS

The nuclear localization and the ability of the *myc* proteins to bind to nucleic acids have led to the hypothesis that they function by directly participating in DNA replication or by transcriptionally activating or down-regulating the expression of target genes. Experimental support has been presented for both these alternatives. Based on the nuclear colocalization of the c-*myc* protein and the small ribonucleoprotein particles (snRNPs), a role for the c-*myc* protein in RNA processing has also been suggested [171]. Of course, it cannot be excluded that the *myc* proteins participate in several of these functions.

Transcriptional Regulation by the *myc* Proteins

By analogy with the viral trans-acting proteins, such as the adenovirus E1A proteins and more recently the cellular oncoproteins c-*fos* and c-*jun,* it has been suggested that the *myc* proteins might also function as transcription factors. When expressed as chimeric fusion proteins containing a heterologous sequence-specific DNA-

binding domain, both c-*myc* and c-*fos* proteins have been found to stimulate transcription in a yeast cell model [230]. However, the significance of the transcriptional activation by the c-*myc* protein in this assay is unclear; its magnitude was only marginal compared to the effect of the c-*fos* protein.

More direct evidence of transcriptional activation by the c-*myc* protein has been presented by Kingston et al. [231,232], who showed that the c-*myc* protein can stimulate transcriptional initiation from the human heat shock protein (hsp70) promoter cloned upstream of a reporter gene. The activity of mouse metallothionein I promoter, in contrast, was found to be downregulated by the c-*myc* protein. Based on corresponding studies, it was recently reported that the c-*myc* protein can trans-activate the adenovirus E4 promoter in embryonal carcinoma cells by using the same target sequences as the E1A products [233]. These studies also suggested that, in contrast to a complete c-*myc* gene construct, a construct devoid of the c-*myc* first exon sequences, and thus capable of producing only the 62 kd human c-*myc* polypeptide, does not have any E4 trans-activating potential.

An interesting finding, with potential biological implications, is the down-regulation of the expression of the class I histocompatibility antigens (MHC I) by N-*myc* expression in rat neuroblastoma cells reported by Bernards et al. [234]. More recently this has also been shown for c-*myc* expression in human melanoma cells [235]. The increased expression of the introduced *myc* gene was found in both studies to correlate with diminished MHC I expression, which could be reversed by treatment of the *myc*-expressing cells with interferon. The molecular mechanism by which the down-regulation of MHC I expression takes place remains, however, unknown. Neither of these studies provided any evidence of direct interaction between the *myc* proteins and DNA elements controlling MHC I gene expression. This is also the case for the novel finding by Schweinfest et al. [236], indicating that under serum-free conditions c-*myc* expression induced from a heat shock promoter up-regulates the expression of two anonymous sequences, previously identified as members of a group of genes rapidly induced by serum [97]. Recently, Prendergast and Cole [237] reported that two sequences, termed *mr*1 and *mr*2 (*myc*-regulated), are specifically induced by an increased c-*myc* expression via a mechanism not involving an enhanced *mr*1 or *mr*2 transcription, suggesting that the c-*myc* protein could up-regulate these genes at some posttranscriptional level.

The potential of the c-*myc* gene to control gene expression may also be reflected by the recently identified short region showing surprising primary structure similarity between the c-*myc* protein and *Myo*D1, a nuclear phosphoprotein triggering myoblast differentiation [238]. Interestingly, deletion of this region of the *Myo*D1 gene, which is also very well conserved between the different *myc* genes, does not affect its nuclear location but eliminates the ability of *Myo*D1 to initiate myogenesis [239].

Stimulation of DNA Replication by the *myc* Proteins

It is generally believed that eukaryotic DNA replication starts with the binding of cellular factors to distinct chromosomal sites from which the replication proceeds bidirectionally; these sites have been termed replication origins (*ori*). The evidence that links the function of the c-*myc* protein to the promotion of the initiation of DNA replication is mostly based on the studies by Iguchi-Ariga et al. [240–242]. A plasmid containing a putative *ori* sequence cloned from mouse liver DNA was shown to be

capable of autonomous replication in mouse and human cells as well as in a cell-free in vitro replication system in the presence of the c-*myc* protein. The c-*myc* protein bound to the putative *ori* sequences of this plasmid. Furthermore, replication of this plasmid could be blocked in vitro by addition of polyclonal or monoclonal c-*myc* antibodies, as well as in intact cells by introducing these antibodies into cells by a liposome-mediated transfection technique [241]. The c-*myc* antibodies were also found to inhibit cellular DNA synthesis when similarly introduced into c-*myc*-expressing leukemia cells, supporting the biological relevance of the *ori*-plasmid studies. Studies by Iguchi-Ariga et al. [240] suggest that the c-*myc* protein also can substitute for the SV40 T antigen in stimulating DNA replication initiated from the SV40 *ori* sequences. Studies by Classon et al. [243] have also demonstrated a facilitated replication of SV40 DNA in c-*myc* expressing cells but did not confirm totally T antigen-independent SV40 replication even in cells expressing high amounts of the c-*myc* protein.

Recent observations suggest that the c-*myc* gene itself has a putative *ori* sequence in its 5′ flanking region. Plasmids containing sequences from the 5′ region of the human c-*myc* gene have been found to be able to replicate autonomously in human cells [242,244]. The c-*myc* protein was shown to bind to these sequences, and, interestingly enough, the same DNA regions were also shown to have transcriptional enhancer activity on heterologous promoters in the CAT assay [242]. Thus the c-*myc* protein may be able to stimulate c-*myc* expression and also to promote the replication of extrachromosomal DNA elements containing the c-*myc* gene, which could have general biological significance, e.g., in the generation of genomic amplifications of the c-*myc* gene in human tumors.

AMPLIFICATION OF *MYC* GENES IN HUMAN LUNG CANCER

Classification of Lung Cancer and Origin of SCLC

The World Health Organization [245] classifies lung cancer into four major histopathological entities: squamous-cell carcinoma (SQC; 25%), SCLC (25%), adenocarcinoma (ADC; 30%), and large-cell carcinoma (LCC; 15%). The remaining 5% consists of uncommon types (such as bronchial carcinoids and combined types).

It is widely, though not unanimously [246], agreed that SCLC arises either from descendants of a pluripotent bronchial stem cell (endoderm derivation), or, in contrast to the other types of lung cancer, from cells originating from the neural crest (ectoderm derivation). The latter hypothesis is based on the neural and neuroendocrine properties of the SCLC cells [43], shared by tissues of the amine-precursor uptake and decarboxylation (APUD) system. However, some of these neuroendocrine properties have also been found to be expressed by non-SCLC cells [247,248]. Furthermore, the original postulate [249] that all cells of the APUD system are derived from the neural crest has since been contradicted [250]. Also, the observations that SCLC tumors may be admixed with or convert to non-SCLC morphologies [251,252] would be difficult to explain if the cellular origin of SCLC is distinctly different from the origin of non-SCLC.

Amplification of the *myc* Genes in SCLC Cell Lines

Amplification, and thereby highly enhanced expression, of the c-*myc* gene has proved to be very common in SCLC cell lines [16,33,34] but may occur as well in cell lines established from non-SCLC tumors [16,253]. In contrast, the high frequency of the

N-*myc* and L-*myc* amplifications in SCLC cell lines [38,44,254,255] is more striking, since amplification or expression of these genes has so far not been encountered in cell lines representing other types of human lung cancer. As a summary of the above studies, it could be estimated that 35–40% of established SCLC cell lines have amplified some of the three well-characterized members of the *myc* gene family. In no case have two different *myc* genes been shown to be amplified to high copy numbers in a single SCLC cell line. No data are available on the possible amplifications of the other, as yet poorly defined, *myc*-related genes.

Minna and collaborators [33,247,256] have described a phenotypic classification of established SCLC cell lines, which shows an association between the amplification of the c-*myc* gene and the biochemical, morphological, and growth properties of these cell lines. In contrast to the "classic" SCLC lines, the variant SCLC lines (SCLC-MV) were found to grow in loosely attached floating aggregates, had a faster doubling time and an increased cloning efficiency in culture, were less sensitive to irradiation in vitro, and failed to express certain biochemical markers typical for SCLC. Only one of the 26 cell lines with a classic morphology disclosed a genomic c-*myc* amplification, whereas seven of the nine SCLC-MV lines had an amplified c-*myc* gene.

An obvious question has been whether the *myc* amplifications have any prognostic significance in SCLC, similar to the well-documented effects of the copy number of the N-*myc* gene on the clinical outcome of neuroblastoma patients [37,257]. Based on the histology of SCLC cell line xenografts in nude mice, Minna et al. [43] proposed that the SCLC-MV lines are derived from SCLC tumors that have unusual, most often LCC-like, morphologies. Such SCLC tumors have been reported to behave more aggressively and to be more resistant to anticancer therapy [258]. However, there is no direct evidence that SCLC tumors with variant morphologies have a higher frequency of c-*myc* amplifications.

Amplification of the *myc* Genes in Lung Cancer In Vivo

To extend the findings on *myc* amplifications in lung cancer cell lines to the in vivo situation, we analyzed DNA from tumor samples from 34 lung cancer patients. Southern blotting analysis of these DNA samples indicated that none of the nine SCLC tumors in this material had amplified c-*myc*, N-*myc*, or L-*myc* [40,44]. The unexpectedly low incidence of *myc* amplifications in clinical SCLC material was then confirmed in other studies [258–262]. A summary of the published data indicates that c-*myc* amplifications have been diagnosed in only two of 114 SCLC tumors from different patients (2%). The frequency of the N-*myc* amplifications in the same material is eight of 114 (7%), whereas the L-*myc* gene has been found amplified in eight of 69 SCLC tumors analyzed (12%). Thus, contrary to what one might expect from a consideration of the in vitro data, c-*myc* amplifications are not frequently found in primary SCLC tumors. In fact, they may be more common in squamous cell carcinoma (SQC) tumors (4/26, 15% in the material cited above). At present, we have no apparent explanation for the observed discrepancy between the in vitro and the in vivo situations. It may be of importance that, in contrast to most SCLC cell lines available, the SCLC samples analyzed by us and by others have been obtained predominantly from primary tumors.

It has been claimed that variant histological features can be seen in less than 10% of SCLC tumors at the time of diagnosis but in more than 30% of autopsy samples [251,263,264], suggesting a selection of the variant morphology during treatment or the

subsequent course of the disease. Also, amplifications of the *myc* genes have been reported to be more common in SCLC cell lines established from tumors from previously treated and relapsed patients than in cell lines derived from primary tumors [254]. In the latter group, the incidence of *myc* amplifications more closely corresponded to that reported for SCLC tumors. Interestingly, in the case of the cell lines from treated and relapsed patients, the presence of a c-*myc* amplification, but not N-*myc* and L-*myc* amplifications, was associated with a shortened patient survival. However, the prognostic significance of *myc* gene amplifications in SCLC tumors is as yet unsettled. Based on an evaluation on in situ hybridization data in a small material of SCLC tumors, Funa et al. [265] have reported that increased expression of the N-*myc* gene is associated with a lack of response to chemotherapy and a poor prognosis. A surprising finding recently reported by Kawashima et al. [266] was a close correlation between restriction fragment length polymorphism (RFLP) of the L-*myc* gene and metastatic properties in a material consisting of various types of human lung cancer.

Unexpectedly, one of the primary lung cancer samples analyzed by us, derived from an adenocarcinoma tumor, gave an intense hybridization signal with the N-*myc* probe [40]. Dilution experiments indicated that the N-*myc* gene was amplified approximately 30-fold in this tumor DNA. This finding shows that amplification of the N-*myc* gene can occur also in non-SCLC and further demonstrates that N-*myc* amplification is not restricted to malignancies classified as neuroendocrine tumors.

It has not been established at which point in the pathogenesis of SCLC the *myc* amplifications develop. Interestingly, studies on experimental gene amplification suggest that anticancer therapy might not only provide a growth selection for *myc* amplified cells but could also promote the generation of these gene amplifications. It has been shown that the rate of drug-resistance gene amplification in vitro may be enhanced up to 1,000-fold by various treatments, including metabolic inhibitors of DNA synthesis, agents that introduce adducts into DNA, and hypoxia, which have in common inhibition of DNA replication [267]. Since anticancer chemotherapy and radiotherapy have similar effects, it might be that the treatment of SCLC patients could promote the development of *myc* amplifications in vivo. On the other hand, high *myc* expression levels might allow the cells with an amplified *myc* gene to survive under the poor growth conditions prevailing during anticancer therapy, resulting in selection of *myc* amplified clones in the tumors at relapse. Both these hypotheses would imply that *myc* gene amplification is a late event in the pathogenesis of SCLC. This idea has not been comprehensively tested, but it is supported by a comparative analysis of *myc* amplifications in primary lung tumors and their metastases reported by Yokota et al. [260]. On the other hand, these data are in conflict with another, corresponding study by Wong et al. [259].

CONCLUSIONS

Deregulation of the members of the *myc* family of oncogenes is involved in many naturally occurring human and animal tumors, and their malignant potential has been convincingly demonstrated by numerous investigations. The active research on the *myc* genes during the past 10 years has produced a large body of knowledge, but our understanding of the precise role of the *myc* genes in normal and malignant growth is still very limited. The cellular factors regulating *myc* gene activity and the biochemical interactions of the *myc* proteins with cellular target molecules are poorly understood.

How do the members of the *myc* gene family differ in these aspects? In which stage of the pathogenesis of the human tumors, such as SCLC, does the *myc* gene deregulation occur, and how should this be taken into account in the clinical work? The ongoing rapid progress in the research on the structural and functional basis of the *myc* gene action would suggest that the answers to these questions will be uncovered in the near future. Let us hope that this information turns out to be more than just another layer in the onion skin, providing us with new questions, but instead improves our understanding of the cellular growth control and offers novel possibilities for intervening in malignant growth.

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