Differential expression of myc, max and RBl genes in human gliomas and glioma cell lines

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Summary Deregulated expression of myc proto-oncogenes is implicated in several human neoplasias. We analysed the expression of c-myc, N-myc, L-myc, max and RBl mRNAs in a panel of human glioma and glioma cell lines and compared the findings with normal neural cells. The max and RBl genes were included in the study because their protein products can interact with the Myc proteins, being thus putative modulators of Myc activity. Several gliomas contained c/L-myc mRNAs at levels higher than those in fetal brain. L-myc was predominantly in grade II/III and c-myc in grade III gliomas. High-level N-myc expression was detected in one small-cell glioblastoma and lower levels in five other gliomas. In contrast, glioma cell lines totally lacked N/L-myc expression. The in situ hybridisations revealed mutually exclusive topographic distribution of myc and glial fibrillary acidic protein (GFAP) mRNAs, and a lack of correlation between myc expression and proliferative activity. max and RBl mRNAs were detected in most tumours and cell lines. The glioma cells displayed interesting alternative splicing patterns of max mRNAs encoding Max proteins which either suppress (Max) or augment (ΔMax) the transforming activity of Myc. We conclude that (1) glioma cells in vivo may coexpress several myc genes, thus resembling fetal neural cells; but (2) cultured glioma cells expression only c-myc; (3) myc, max and RBl are regulated independently in glioma cells; and (4) alternative processing of max mRNA in some glioma cells results in ΔMax encoding mRNAs not seen in normal fetal brain.

Proto-oncogenes relay signals that regulate cell growth and differentiation, and their aberrant activation is implicated in a wide spectrum of neoplasia. The human myc proto-oncogenes (c-myc, N-myc and L-myc) encode related DNA-binding phosphoproteins that presumably function as transcriptional regulators of specific target genes (Lüscher & Eisenman, 1990). The Myc proteins can bind DNA alone, but more effectively in a complex with Max, a heterodimeric Myc DNA-binding partner (Blackwood & Eisenman, 1991). Max contains a DNA-binding basic region (b) N-terminal to helix-loop-helix (HLH) and leucine zipper (Zip) dimerisation motifs. Dimerisation with Max increases the capability of Myc to bind DNA in a sequence-specific manner (Blackwood & Eisenman, 1991), and modulates the transforming activity of Myc (Prendergast et al., 1991). Max associates with N-Myc in vivo in neuroblastoma cells (Wenzel et al., 1991), and with c-Myc, N-Myc as well as L-Myc in rat embryo fibroblasts (Mukherjee et al., 1992). This interaction appears to be a common mechanism through which Myc function is mediated (Mukherjee et al., 1992). Several different forms of Max are generated through alternative mRNA splicing: Max suppresses, while the C-terminally truncated ΔMax enhances, Myc transformation (Mäkelä et al., 1992; Västrik et al., 1993). These opposing effects of Max and ΔMax on the transforming activity of Myc suggest that max mRNA processing may be an important mechanism to modulate Myc activity. Intriguingly, the N-termini of c-Myc and N-Myc bind to the retinoblastoma (RBl) protein in vitro (Rustgi et al., 1991). Inactivation of the RBl gene is strongly implicated in the pathogenesis of childhood retinoblastomas and in sporadic tumours of other cell lineages as well (Gallie et al., 1990). The RBl protein may thus, in addition to Max, be a modulator of Myc activity, although the interaction has not been demonstrated in vivo.

High-level myc expression releases several cell types from growth-regulatory constraints, e.g. by reducing their growth factor requirements for continuous proliferation and by causing a dedifferentiation to a more primitive phenotype, thereby contributing to a neoplastic phenotype (Lüscher & Eisenman, 1990; DePinho et al., 1991). Genomic amplifications of N-myc or L-myc and consequent high-level mRNA and protein expression are frequent in tumours displaying neuroectodermal characteristics, particularly in neuroblastomas (N-myc) (Kohl et al., 1984; Schwab et al., 1984) and small-cell lung carcinomas (SCLC) (N/L-myc) (Nata et al., 1985, 1986; Wong et al., 1986; Johnson et al., 1988). c-myc amplifications are also frequent in several other tumour types (Altitalo et al., 1987).

The genetic events involved in CNS tumorigenesis have recently been reviewed (James & Collins, 1992). Among the best-characterised alterations is the frequent amplification and overexpression of the EGF receptor gene (Libermann et al., 1985), but sporadically amplified c-myc (Trent et al., 1986, N-myc (Bignier et al., 1988; Fujimoto et al., 1989) and c-myb (Welter et al., 1990) genes have also been described in glioma cells.

We have previously characterised the developmental expression of the three myc genes and the alternative mRNA processing of L-myc in fetal brain (Hirvonen et al., 1990). We found that L-myc, N-myc as well as c-myc expression is not coupled to mitotic activity in fetal brain, suggesting that myc expression is characteristic for an immature phenotype rather than cell proliferation. Bearing in mind that malignant tumours often overexpress the same proto-oncogenes that are active during the normal embryonic development and growth of the cells of origin of the tumour, we have now extended these analyses to human central nervous system (CNS) malignancies, in order to compare the expression of myc genes in malignant and normal neuroepithelial cell growth. We now report differentially regulated (co)expression of the myc genes (c-M, N- and L-myc), and the max and RBl genes (encoding putative modulators of Myc activity), in a panel of human brain tumours and glioma cell lines, using Northern hybridisation, RNAase protection and, in selected cases, in situ hybridisation. The findings are compared with normal fetal brain as well as with the expression of well-established neuronal and glial marker genes: mid-weight neurofilament subunit (NF-M) specific for neurons, and glial fibrillary acidic protein (GFAP) and vimentin, which within the CNS is specific for glial cells.
Materials and methods

Brain tumour specimens

Primary brain tumour samples (n = 13) were obtained at neurosurgical operations. The normal adult white and grey matter specimens were obtained from a patient operated on for an arteriovenous malformation, with no disorder affecting the white or grey matter. Human fetal brain specimens were obtained from therapeutic second-trimester abortuses as described previously, with approval of the ethical committee of Turku University Central Hospital (Hirvonen et al., 1990). These were chosen as controls because they represent the normal counterparts of the tumour cells, albeit they consist of heterogeneous cell populations. The tissue pieces were divided into three specimens: one of two adjacent samples was snap-frozen in liquid nitrogen immediately upon removal for the isolation of RNA and DNA, while the other was sent to the Department of Pathology for diagnostic frozen section. The third part of the samples was fixed in phosphate-buffered 4% formaldehyde and processed to routine paraffin specimens to be used in the final diagnostics and in situ hybridisation analyses. The tumours were classified according to the WHO International Histological Classification of Tumors No. 21 (Zülch, 1979). The grading of astrocytomas and glioblastomas that was used corresponds closely to that of the St Anne-Mayo system (Daumas-Dupont et al., 1988), i.e. grade I, no or minimal cellular atypia and low cell density; grade II, some cellular atypia and higher cell density; grade III, cellular atypia, mitoses, vascular proliferation; glioblastoma, marked atypia, mitoses, necroses. The diagnosis of small-cell glioblastoma (SCGB) was ascribed to a tumour, which first presented as a grade III astrocytoma and upon recurrence 1 year later (analysed in this study) disclosed a microscopic picture dominated by tightly packed small, actively dividing cells with intervening anaplastic astrocytic cells. A summary of the tumours is given in Table I.

Glioma cell lines

The human glioma cell lines U-118 MG, U-251 MG, U-343, U-410, U-251 MG-Sp, U-178 MG and U-1242 MG were a kind gift from B. Westermark, Uppsala, Sweden. The glioma lines A-172, T98G, U-87 MG, U-138 MG, U-373 MG, HS 683 and the neuroglioma line H4 were obtained through the ATCC. The cells were cultured in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

Isolation and analysis of RNA

Total cellular RNAs were isolated as previously described (Chirgwin et al., 1979). The tumour specimens were homogenised in 4 M guanidinium isothiocyanate (GIT) and the RNAs purified by ultracentrifugation through 5.7 M caesium chloride cushions. From the glioma cell lines total RNA was isolated by lysing confluent cultures directly into GIT; fresh medium was added to the cultures 24 h prior to RNA isolation, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) before the GIT lysis. Aliquots of 15 μg total cellular RNAs were size fractionated in 1% agarose/formaldehyde gels, blotted onto nylon membrane (Genescreen Plus, DuPont, NEN, MA, USA), and hybridised under stringent conditions according to the manufacturer’s suggestions. The sequential probing of the filters was carried out with intervening removal of the previous probe as suggested by the manufacturer.

Table I Summary of mRNA analyses in the astrocytoma samples

| Specimen          | PAD/grade | c-myc | N-myc | L-myc | max | RBl | GFAP | VIM | NF-M |
|-------------------|-----------|-------|-------|-------|-----|-----|------|-----|------|
| Normal adult cortex | -         | (+)   | -     | -     | +   | -   | -    | -   | -    |
| Normal adult white matter | -     | -     | -     | -     | +   | +   | +    | +   | -    |
| Fetal brain       | +         | +     | +     | +     | +   | -   | +    | -   | +    |
| No. 1 AC I        | +         | -     | +     | +     | +   | +   | +    | +   | +    |
| No. 2 AC I        | -         | +     | +     | +     | -   | +   | +    | +   | +    |
| No. 3 GN          | +         | +     | +     | +     | +   | +   | +    | +   | ++   |
| No. 4 AC II       | +         | -     | +     | +     | +   | +   | +    | +   | +    |
| No. 5 AC II       | +         | +     | +     | +     | +   | +   | +    | +   | +    |
| No. 6 A11         | ND        | ND    | +     | ND    | ND  | ND  | ND   | ND  | ND   |
| No. 7 AC III      | + +       | -     | +     | +     | +   | +   | +    | +   | +    |
| No. 8 AC III      | +         | +     | +     | +     | +   | -   | +    | +   | +    |
| No. 9 AC III      | +         | +     | +     | +     | +   | +   | +    | +   | +    |
| No. 10 GB         | + + +     | +     | +     | +     | +   | +   | +    | +   | +    |
| No. 11 GB         | + + +     | +     | +     | +     | +   | -   | +    | +   | +    |
| No. 12 GB         | +         | +     | +     | +     | +   | +   | +    | +   | +    |
| No. 13 SCGB       | ++        | + +   | +     | +     | +   | -   | +    | +   | +    |
| No. 14 NB IV      | +         | +     | +     | +     | +   | -   | +    | +   | +    |
| No. 15 NB IV      | +         | +     | +     | +     | +   | -   | +    | +   | +    |
| No. 16 SCLC       | + + +     | +     | +     | +     | +   | +   | +    | +   | +    |
| No. 17 CNS lymphoma | +       | +     | +     | +     | +   | -   | +    | +   | +    |

The following symbols denote the mRNA levels in an increasing order: –, +, ++, +++, +++++. Of the glioblastomas, nos. 11 and 12 are of the classic type with pseudopalisadic necroses, while no. 13 is the PNET-like small-cell glioblastoma (SCGB). c-myc analyses carried out by Northern hybridisation only, not by RNAase protection, owing to paucity of RNA. Abbreviations: GFAP, glial fibrillary acidic protein; VIM, vimentin; NF-M, mid-weight neurofilament subunit; AC, astrocytoma; GN, ganglioglioma; GB, glioblastoma; SCGB, small-cell glioblastoma; NB, neuroblastoma; SCLC, small-cell lung carcinoma (CNS metastasis).
lated and purified from agarose gels by isotachophoresis (Öfverstedt et al., 1984), and labelled by the random-priming method (Feinberg & Vogelstein, 1983) with [α-32P]dCTP (Amersham, UK) to specific activities of 1–2 x 10⁶ c.p.m. µg⁻¹. The unincorporated nucleotides were removed in Sephadex G50 spin columns.

**RNAase protection assays**

For RNAase protection assays, the cRNA probes were generated from linearised templates (see above) using commercial transcription kits (Transprobe SP and T, Pharmacia, Sweden) with [α-32P]UTP (Amersham, UK). In some experiments, the L-myc (pL2Gem450) and RBI probes were combined in order to use the RBI signal as an internal control. The N-myc and c-myc cRNA probes (protected as 353- and 316-nt fragments respectively) were not mixed with the RBI probe to avoid possible blurring of the myc signals by an eventual residual RBI probe (322 nt) observed in some experiments. Synthesis and purification of the probes and the hybridisations were carried out as described previously (Hirvonen et al., 1990).

Figure 1 Northern hybridization analyses of c-myc, N-myc, max, neurofilament-M (NF-M), vimentin (Vim) and GFAP mRNAs in human CNS tumours. For controls, RNAs from normal fetal brain and normal adult grey (GM) and white (WM) matter were included. The sizes of the transcripts are indicated on the right. Ethidium bromide (EtBr)-stained RNAs are shown below, with the 28S and 18S rRNAs marked.
**Figure 2** RNAase protection analysis of *myc* mRNAs in human malignant gliomas and fetal brain. The sizes of the cRNA probes are shown to the left of each panel. The sizes of the protected fragments are 316 nt (c-My), 353 nt (N-My) and 421 and 457 nt (L-My). The two L-My fragments (421 and 457 nt) are protected by alternatively spliced L-My transcripts. Analysis of N-My mRNA was carried out to verify the weak in situ hybridisation signals (see Figure 5) obtained in some tumours which in Northern hybridisation lacked detectable N-My mRNA (see Figure 1). The 419-nt band in the N-My panel results from residual incompletely digested probe.

**Figure 3** RNAase protection analysis of *myc* and *RB1* mRNAs in glioma cell lines. c-My and RB1 but not N-My or L-My probes are protected by glioma cell RNAs. The fetal brain RNA used as positive control (the rightmost lane) is slightly degraded. Therefore this RNA yields a relatively stronger signal in the RNAase protection than in the Northern analysis (cf. Figure 1).
In situ hybridisations

The in situ hybridisations were carried out on 5-µm-thick sections of formaldehyde-fixed, paraffin-embedded material with [α-35S]ATP-labelled probes (Sandberg & Vuorio, 1987). As a negative control probe we used bacteriophage λ DNA fragments of 100–790 bp generated by BglI. After stringent washes the dried slides were dipped into autoradiography emulsion (Kodak, NTB 3), exposed for 3–12 weeks, developed and counterstained with haematoxylin.

Results

myc and max mRNA expression in brain tumours

Northern hybridisations revealed c-myc expression at levels higher than in adult brain in 10 out of 13 surgically removed gliomas. The c-myc signal intensities were stronger in the high-grade (III and IV) and weaker in the low-grade gliomas. However, one grade IV and one grade I tumour lacked c-myc signals. Enhanced N-myc expression was detected by Northern hybridisation in only one tumour, a highly malignant small-cell glioblastoma (SCGB), in addition to the stage IV neuroblastoma used as a positive N-myc control (Figure 1). The N-myc copy number in this tumour appeared normal as analysed by Southern hybridisation (not shown). However, the in situ hybridisations revealed N-myc-reactive cells in some other gliomas after long exposure times (see below). This finding was verified by the more sensitive RNase protection analysis, which indeed revealed definite N-myc signals in five gliomas (Figure 2). These signals were several-fold stronger than the very weak signal observed in the adult brain RNA.

Elevated L-myc mRNA levels (compared with fetal brain) were detected in 5 out of 13 tumours (Figure 2). The highest L-myc mRNA levels were observed in the grade II/III astrocytomas and the SCGB. The two protected L-myc fragments (421 and 457 nt) indicated that at least two L-myc mRNA forms (either lacking or retaining intron I) were present. Further RNAase protection analyses revealed that the majority of L-myc transcripts contained exon III (data not shown). Thus, only the two long L-myc mRNA splice variants (3.6 kb and 3.8 kb) were present, but not the short transcripts which lack exon III and which have been detected in SCLC cell lines and some leukaemia cells only (Kaye et al., 1988; Hirvonen et al., 1991). The RBI mRNA, which is expressed at almost uniform levels in normal cells and tissues, was detectable in all tumours as well as in fetal and adult brain (shown for glioma cell lines in Figure 3).

Hybridisation with the max probe yielded signals in 9 out of 13 glioma tumours. The overall expression patterns of max did not parallel those of c-myc, N-myc or L-myc (Figure 1). Five different max mRNAs have been identified, with sizes of 1.9 kb, 2.3 kb, 2.4 kb, 3.0 kb and 3.5 kb. The major 2.3-kb and 2.4-kb mRNAs encode for Max and ΔMax respectively (Västrik et al., 1993). In some tumours, additional bands of 1.9 kb, 3.0 kb and 3.5 kb were observed. The 3.0-kb mRNA codes for ΔMax, and the 3.5-kb mRNA for a ΔMax-related polypeptide, which lack the nuclear targeting signal (Västrik et al., 1993). The 1.9-kb and 2.3-kb mRNAs encode ‘normal’ Max (Blackwood & Eisenman, 1991). These max mRNA processing patterns differed from those seen in normal fetal brain (see Figures 1 and 4 and Table II).

Probes for cell type-specific intermediate filament mRNA were analysed to confirm and further characterise the histopathological classification of the tumours and glioma cell lines. Vimentin mRNA was detected in all tumours and cell lines, as well as in fetal but not in adult brain. GFAP mRNA was present in most tumours but in only four glioma cell lines. Normal adult brain white matter gave a GFAP signal, while fetal brain and normal adult grey matter yielded only faint signals after very long exposure times (not visible in Figure 1). The mid-weight neurofilament subunit (NF-M) mRNA was expressed in adult grey matter, fetal brain and in some low-grade gliomas, but not in adult white matter. In addition, NF-M mRNA was detected in the grade IV neuroblastoma and in the SCGB tumour. The one primary CNS lymphoma and the SCLC brain metastasis lacked GFAP and NF-M mRNAs, consistent with their non-glial and non-neural origin (Table I).

![Figure 4](image)

**Table II** Alternative splicing of max mRNAs in glioma cells

| Cell line | Max mRNA size (encoded polypeptide) | Max mRNA (Max) | ΔMax (ΔMax) | ΔMax + ΔMax |
|-----------|------------------------------------|----------------|-------------|-------------|
| A-172     | +                                  | +              | −           | −           |
| T98G      | −                                  | −              | −           | +           |
| U-87 MG   | −                                  | + +            | +           | +           |
| U-87 MG (f)| −                                   | +              | −           | +           |
| U-138 MG  | +                                  | + +            | −           | −           |
| U-373 MG  | +                                  | + +            | −           | −           |
| Hs 683    | −                                   | (−)            | +           | +           |
| H4        | −                                  | −              | −           | (−)         |
| U-118 MG  | −                                  | −              | −           | (−)         |
| U-251 MG  | −                                  | +              | −           | (−)         |
| U-251 MG-Sp| +                                  | + +            | −           | −           |
| U-343 MG  | +                                  | + +            | −           | −           |
| U-410 MG  | −                                  | −              | −           | (−)         |
| U-178 MG  | −                                  | −              | −           | (−)         |
| U-1242 MG | +                                  | + +            | −           | (−)         |

Fetal brain

(+) + + − −

The following symbols denote semiquantitation of the levels of the different max mRNA forms, in increasing order: −, +, + +, + + +. The 2.3-kb and 2.4-kb mRNAs encoding Max and ΔMax, respectively, migrate very closely therefore they are listed together in the table as the 2.3-kb column. The 3.5-kb transcript encodes a variant ΔMax protein (Västrik et al., 1993).
Expression of myc, max and RBl mRNAs in glioma cell lines

In the glioma cell lines, the N-myc, L-myc and RBl mRNAs were analysed by RNAase protection, while max, neurofilament-M, GFAP and vimentin mRNA expression analyses were carried out by Northern hybridisations. Non-coordinate expression patterns of c-myc and max were evident in the cell lines. For example, T98G cells yielded a very strong c-myc signal but almost entirely lacked max mRNA except a very weak 3.5-kb band, corresponding to a variant ΔMax (Väistrik et al., 1993). U-373 MG cells showed strong max signals but only a weak c-myc signal, while U-1242 cells contained both intense max and c-myc signals (Figure 4). All the glioma cell lines lacked entirely N-myc and L-myc mRNAs as analysed by RNAase protection (Figure 3). The strong c-myc signal observed in fetal brain in these protection analyses (Figure 3, rightmost lane) contrasts with the relatively weaker c-myc signal obtained in the Northern analysis of the same specimen (Figure 3, rightmost lane). This is due to partial degradation of this particular RNA sample, which results in the disproportionately weaker Northern signal in Figure 4. However, the 316-nt c-myc cRNA probe can be efficiently protected even by the partially degraded c-myc mRNA. With the exception of the H4 neuroglioma cell lines, all the cell lines yielded strong vimentin signals, while GFAP mRNA was detectable only in U-373, U-251 MG, U-251 MG-Sp and U-178 MG cells. None of the cell lines contained NF-M mRNA as analysed by Northern hybridisation. These expression data are summarised in Table III. As in the tumours, max mRNA processing patterns exhibited cell line-specific variation, resulting in the production of Max and ΔMax-encoding mRNAs at varying proportions. Data on these alternative max mRNA splice variants are summarised in Table II.

Table III Summary of mRNA analyses in the glioma cell lines

| Cell line | c-myc | N-myc | L-myc | max | RBl | GFAP | VIM | NF-M |
|-----------|-------|-------|-------|-----|-----|------|-----|------|
| A-172     | ++    | -     | -     | +   | +   | -    | +   | -    |
| T98G      | + + + | -     | -     | -   | +   | +    | +   | -    |
| U-87 MG   | + + + | -     | -     | +   | -   | +    | +   | -    |
| U-87 MG (f) | + + + | -     | -     | +   | -   | +    | +   | -    |
| U-138 MG  | + + + | -     | -     | + + | -   | +    | +   | + +  |
| U-373 MG  | +     | -     | -     | + + | +   | +    | +   | +    |
| Hs 683    | +     | -     | -     | +   | -   | +    | +   | -    |
| H4        | +     | +     | +     | +   | -   | +    | +   | -    |
| U-118 MG  | + + + | -     | -     | +   | +   | +    | +   | -    |
| U-251 MG  | +     | -     | -     | +   | -   | +    | +   | -    |
| U-251 MG-Sp | +     | -     | -     | +   | -   | +    | +   | -    |
| U-343 MG  | +     | -     | -     | +   | +   | +    | +   | -    |
| U-410 MG  | +     | -     | -     | +   | -   | +    | +   | -    |
| U-178 MG  | -     | -     | -     | +   | +   | +    | +   | -    |
| U-1242 MG | +     | -     | -     | +   | -   | +    | +   | -    |
| Fetal brain | + +  | +     | +     | +   | -   | +    | +   | -    |

The following symbols denote semiquantitation of the mRNA levels in increasing order: −, +, ++, ++++, +, ++ +, +++. N/L-myc and RBl mRNAs were analysed by RNAase protection only, c-myc by Northern hybridisation and RNAase protection, and vimentin, GFAP and NF-M by Northern hybridisation only. For max, the semiquantitation given indicates presence of some of the several max mRNAs generated via alternative splicing; these are listed separately in detail in Table II. U-87 MG(f) = U-87 MG cell spheroids shed into the culture medium.

Localisation of myc and GFAP mRNAs by in situ hybridisation

In selected gliomas, in situ hybridisation was utilised to localise and identify cells expressing myc and GFAP genes. Great topographic variation was observed in the myc hybridisation signal intensities. The myc autoradiographic grains were localised over malignant cells, but the strongest signals did not co-localise with regions of the highest mitotic activity. The glioblastoma with the highest c-myc mRNA levels (no. 10) displayed accentuated c-myc signals over some but not all malignant cell clusters, and in particular over pseudopalisadic tumour cell formations surrounding the necrotic lesion. In contrast, the reactive endothelial cell proliferations lacked a c-myc signal (Figure 5a–d). A few glioma cells also reacted with the N-myc probe, but the signal intensities were much weaker than with the c-myc probe. This weak N-myc signal was further confirmed by RNAase protections (see above: compare Figures 1 and 2). We observed an inverse topographic localisation of the GFAP and c-myc hybridisation signals: the cells with the highest c-myc expression showed only a weak or no GFAP signal (compare Figure 5b and d), while strongly GFAP-positive cells lacked c-myc mRNA (compare Figure 5f and h).

The SCGB tumour (no. 13) which had very high N-myc mRNA levels, displayed a non-identical topographic distribution of N-myc, L-myc and c-myc mRNA signals. The tumour cells displayed heterogeneous N-myc signal with some very intensely labelled tumour cell clusters, which lacked c/L-myc RNA (Figure 6a–e). In contrast, within areas with the highest proliferative activity only scattered cells contained N-myc mRNA. Areas containing reactive astrocytes mixed among malignant cells yielded positive hybridisation signals with c-myc, N-myc and L-myc probes, but not with entirely similar distribution patterns (Figure 6f–j). Thus, subpopulations of cells with differentially activated myc expression exist within this rare tumour.

Discussion

Several glioma tumours contained N-myc and L-myc mRNAs as detected by RNAase protection, in addition to the high c-myc mRNA levels present in most glioma tumours and cell lines. Some tumours and most cell lines exhibited strong max mRNA signals, with evidence of differential alternative splicing. RBl mRNA was detected in all the gliomas, suggesting that RBl deletions play no role in glial cell transformation. However, subtle mutations abolishing the production of a normal RBl protein cannot be detected by the methods used. Vimentin mRNA was detectable in all the tumours and cell lines, GFAP in most glioma tumours but in only four glioma cell lines. NF-M mRNA was absent from the cell lines and present in only a few low-grade gliomas, the signals most probably originating from neurons within the tumour tissue. No definite clinical characteristics could be assigned to different myc or max expression profiles in this limited set of patients. However, the higher c-myc mRNA levels and simultaneous presence of N-myc and L-myc mRNAs paralleled a higher grade of malignancy in astrocytomas grade I–III, whereas in the grade IV classic glioblastomas N-myc and L-myc were not active. The enhanced myc expression probably results from increased transcription or prolonged
mRNA half-life, since we did not detect amplified myc genes in our tumour panel as analysed by Southern hybridisation.

Enhanced expression of the N-myc gene has been implicated in the pathogenesis of extracranial tumours with neuroectodermal characteristics, such as peripheral neuroblastomas and some cases of SCLC (DePinho et al., 1991). In neuroblastomas, N-myc amplification is a strong indicator of poor prognosis, independently of other staging criteria (Brodeur et al., 1984; Seeger et al., 1985). Our results show that some glioma tumours may express N-myc mRNA simultaneously with c-myc and/or L-myc. The N-myc mRNA levels in these tumours are detectable by RNAase protection but not by Northern hybridisation. The partially overlapping but non-identical topographic distribution patterns of the myc in situ hybridisation signals are suggestive of differential regulation of the genes in glial cells. The high levels of N-myc in the SCGB differed sharply from other high-grade gliomas. The histopathological picture of this tumour was dominated by

Figure 5 In situ hybridisation analysis of c-myc, N-myc and GFAP mRNA. a–d and e–h, respectively, represent the same fields of serial sections of a glioblastoma (tumour no. 10) hybridised with the different probes. a–d, A strong c-myc signal is seen in b over malignant cells surrounding the necrotic area (n) and the endothelial cell proliferation (en), while most c-myc-positive cells remain GFAP negative (d). Some c-myc-reactive cells also yield a weak N-myc signal (e). This N-myc signal was verified by RNAase protection analyses (Figure 2), although in Northern hybridisation this tumour appeared devoid of N-myc mRNA. e–h, An opposite topographic distribution is evident for the c-myc vs GFAP signals. The reactive astrocytes in this field show a very strong GFAP signal (h), only an extremely weak c-myc signal (f), and no N-myc signal (g). Bar = 100 μm.
Figure 6 Differential localisation of myc mRNAs in the small-cell glioblastoma tumour (SCGB). b, A strong N-myc signal in tumour cells that lack c-myc (c), L-myc (d) and GFAP (e) mRNAs. a, A bright-field image of the same field shown in b–e. f–j, Tumour cells mixed among few reactive astrocytes show scattered, but not strictly identical distribution, patterns of c-myc (b), N-myc (g) and L-myc (i) mRNAs, but lack of GFAP mRNA (j). Bar in a (for a–e) and f (for f–j) = 200 μm.

undifferentiated small cells identical to those characteristic of primitive neuroectodermal tumours (PNETs). This, together with the presence of NF-M and vimentin mRNA and the lack of GFAP mRNA, makes it difficult to assign a specific diagnosis for this tumour. The diagnosis of PNET with neuronal and glial differentiation would be justified for this rare tumour, but since PNET terminology is somewhat controversial the diagnosis of SCGB has been adhered to here.
The finding prompts further investigations as to whether high-level N-myc expression could be a useful marker for malignant small-cell tumours in the brain, i.e. for the disputed PNET group (Rorke, 1983), particularly if complemented with other cell type-specific markers of tumour differentiation and stage.

L-myc expression has not been previously reported in gliomas. In our tumour material, several gliomas contained L-myc mRNAs at levels equal to or in excess of those in fetal brain. This underlines the usefulness of sensitive RNase protection analyses rather than ordinary Northern hybridisation in studies of low-abundance mRNAs like L-myc. Even though L-myc (and N-myc) mRNAs were present in most grade III malignant astrocytomas, they were absent from two classic glioblastomas. In contrast, the glioma cell lines adapted to growth in vitro lacked L-myc and N-myc mRNAs, but most of them contained high-levels of c-myc mRNA. This finding is consistent with earlier observations of a lack of N-myc and L-myc mRNAs and presence of c-myc mRNA in glioma cell lines (LaRocca et al., 1989).

In situ hybridisations revealed the highest myc mRNA levels in regions distinct from those with the highest proliferative activity. This finding parallels our observations and those of others of uncoupling of N-myc (Grady et al., 1987; Hirvonen et al., 1989) as well as c-myc and L-myc (Hirvonen et al., 1991a,b) from mitotic activity in fetal brain. In gliomas, the high myc mRNA levels may be linked to a dedifferentiated state rather than proliferative activity, as is the case in developing fetal CNS. This notion is supported by the finding that GFAP and c-myc mRNAs display mutually exclusive topographic distribution patterns in gliomas as analysed by in situ hybridisation. Similarly, the glioma cell lines with the highest c-myc expression levels were negative for GFAP mRNA but did contain vimentin mRNA, which is the earlier of the two intermediate filaments expressed during glial cell differentiation.

Several glioma cells and tumours contained max mRNA at levels equal to or in excess of those found in normal human fetal brain. max mRNA levels in glioma cells are regulated independently of the myc genes, and show great variation between different cell lines. Interestingly, the max mRNA processing patterns varied between different glioma tumours and glioma lines. This may be of functional importance, since alternative max mRNA processing results in at least five different max mRNAs with different protein-coding capacities and opposing effects on the transforming activity of Myc. The first identified, Max (Blackwood & Eisenman, 1991), suppresses while the C-terminally truncated ΔMax augments Myc transformation (Mäkelä et al., 1992). In addition to the major 2.3- and 2.4-kb bands representing Max- and ΔMax-encoding mRNAs, respectively, several glioma cells contained additional max mRNAs of 1.9 kb, 3.0 kb and 3.5 kb. Recent data indicate that the 3.0-kb and 3.5-kb max mRNAs encode Max polypeptides that are structurally identical (3.0 kb) or highly similar (3.5 kb) to ΔMax, and which enhance the transforming activity of Myc in Myc–Ras co-transformation assays (Västrik et al., 1993). The amount and composition of the different Myc–Max heterodimers may thus be regulated via max transcription and mRNA splicing, in addition to the regulation of myc mRNA levels. In K562 cells, a pH decrease causes a switch from the 2.3- and 2.4-kb max mRNAs to the 3.5-kb mRNA form, suggesting that acidification of the culture medium may regulate max mRNA processing (Västrik et al., 1993).

In the case of the glioma cells analysed here we consider this an unlikely source of max mRNA splice pattern variation between different cell lines, as all the cultures were confluent and medium was changed 24 h prior to RNA extraction. Further studies will undoubtedly clarify the functional significance of the different Max forms in normal and malignant neuroectodermal cells and their derivatives.

We have previously reported combinatorial expression of several myc genes in human leukemias and leukemica cell lines (Hirvonen et al., 1991). Our present results suggest that some gliomas may coexpress several myc genes, thus in part augmenting the restricted tissue spectrum of N-myc and L-myc expression. It is possible that L-myc and N-myc are transiently activated in some early stages of the transformation of glial cells and contribute to their escape from growth restrains. In this context it is interesting that susceptible N-myc-expressing glioma cells which undergo the phenotypic instability manifested as a transdifferentiation potential, which can be abolished by blocking N-Myc production using antisense N-myc expression constructs (Whitesell et al., 1991). The discrepancy between L-myc and N-myc expression in gliomas in vivo as opposed to glioma cells in vitro does not result from eventual normal cells trapped within the biopsy specimens, as in the in situ analyses the L-myc and N-myc signals were located over neoplastic gial cells. It is possible that culture conditions select for the glioma cells with the highest c-myc expression levels, which conceivably would result in a cell population with silent N-myc and L-myc genes, as a result of negative myc cross-regulation. Since c-myc but not N- or L-myc appears to be involved in the signalling pathways of several growth factors, c-myc expression might best allow the glioma cells to circumvent the lack of exogenous growth factors in culture. Further analysis of myc and max in normal CNS cells and identification of their target genes in glioma cells will undoubtedly be helpful in establishing their roles in normal and malignant glial cell growth.

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