Expression, regulation, and chromosomal localization of the Max gene

(Myc/cell growth/differentiation/protooncogene/transformation)

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ABSTRACT The Max gene encodes a protein that interacts specifically with the Myc protein to form a heterodimer with high affinity for the specific cognate DNA binding site of Myc. Here we examine the expression of Max RNA in comparison to Myc RNA during cell growth and differentiation. Two species of RNA, a major 2.0- and a minor 1.7-kilobase species, hybridized specifically to a Max cDNA probe in all human and murine cell lines that were tested. Unlike Myc, the steady-state level of Max RNA is not significantly modulated with respect to proliferation or differentiation. Max RNA is expressed in quiescent BALB/c 3T3 cells and is modestly increased 3 h after addition of serum to the quiescent cells. In contrast to Myc RNA, Max RNA does not decline immediately upon induction of differentiation of HL60 cells by dimethyl sulfoxide, and only a modest decrease of Max RNA was observed 72 h after induction of differentiation. Unlike Myc RNA, Max RNA is relatively stable with a half-life of >3 h and, therefore, does not exhibit the characteristic short half-life of RNAs encoded by most immediate early genes. The human Max gene was localized to chromosome 14, band q23. With respect to the recurring abnormalities in human tumors, this region of chromosome 14 is involved in deletions in B-cell chronic lymphocytic leukemia and malignant lymphomas and in the 12;14 translocation in uterine leiomyomas.

The processes of cell proliferation and differentiation are closely correlated with changes in the level of expression of the protooncogene *c-myc* (for reviews, see refs. 1 and 2). During the induction of cell growth by the addition of growth factors and mitogens to quiescent cells, the steady-state level of Myc RNA is transiently increased and remains at relatively high levels in proliferating cells (3–5). During cellular differentiation, however, the level of Myc RNA expression decreases as cells enter a nonproliferative state (6–8). Furthermore, it is well documented that deregulation of Myc expression is associated with tumorigenesis (1, 2, 9, 10).

Despite much data suggesting the importance of Myc in these events, little is known about the mechanism of its action. The similarity of the predicted structure of the protein's domains with other transcription factors and its nuclear localization suggest that it may regulate transcription of other genes (11). The Myc protein contains a DNA-binding basic domain coupled to a helix-loop-helix motif adjacent to a leucine-zipper region; both the helix-loop-helix and the leucine-zipper domains are characteristic of protein dimerization domains (11–13). On the N-terminal side of these basic helix-loop-helix/leucine-zipper domains lies an acidic domain characteristic of activating domains. This region of Myc has been shown to have activating capabilities in the form of a fusion protein with the DNA binding domain of GAL4, a yeast transcriptional activator (14). Identification by

Blackwell *et al.* (15) of a hexanucleotide DNA sequence as the specific DNA binding site for Myc, albeit only at high protein concentration, has allowed additional speculation that indeed Myc can serve as a transcriptional regulator. Furthermore, support for this role has also been found in the recent discoveries of Max and Myn, two highly homologous proteins found in human (16) and murine (17) cells. The human Max gene and its murine homologue, Myn, were identified on the basis of the ability of their gene products to dimerize with Myc and to form a DNA binding complex with higher affinity for the Myc-binding site than Myc alone.

The predicted tertiary structure of Max (this term hereafter refers to both Max and Myn) closely resembles that of Myc, and it was on the basis of the basic/helix-loop-helix/leucine-zipper homology that Prendergast *et al.* (17) were able to clone the cDNA encoding Max. The role of Max is unclear, but it may serve to modulate Myc activity in a positive (17) or negative (18) manner. In this regard, the regulation of Max expression relative to Myc may play an important role in the control of cell growth and differentiation.

The interaction between Myc and Max and the possible importance of the relative levels of the two proteins *in vivo* led us to study the patterns of expression of these two genes in relation to each other in proliferating and differentiating cells and in tumor cells with deregulated Myc expression. Here we report that Max RNA is relatively stable and that its expression is modestly induced after serum stimulation of resting cells and not significantly altered during cellular differentiation.

MATERIALS AND METHODS

Cell Culture. HL60 (a human myeloid cell line); the Burkitt lymphoma cell lines Ramos, ST486, PA682, and EB-2; BJAB and RPMI 1788 (human B-cell lines); and WEHI-231 (a murine immature B-cell line) cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. Human skin fibroblasts and BALB/c 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

For serum stimulation experiments, quiescent BALB/c 3T3 cells were maintained in 0.5% fetal calf serum for 24 h and then stimulated for various times with 20% fetal calf serum. HL60 differentiation was accomplished by adding 1.5% (vol/vol) dimethyl sulfoxide (DMSO) to cells for various times.

The PCR of Max cDNA. A partial Max cDNA was obtained by using the PCR, a human testis cDNA recombinant phage λ gt11 library (Clontech), and primers derived from the sequence published by Blackwood and Eisenman (16). MAX1, the 5' primer, had the sequence CCTGGGCCGTAGGAAAT-

GAGCGATAAC and included the presumed translation start site of the open reading frame (indicated in boldface type). MAX2, the 3' primer, had the sequence TGCCAGTGGCT-TAGCTGGCCTCCA and included the end of the open reading frame (indicated in boldface type). Twenty-five cycles of the PCR were performed at 95°C for 1 min, 65°C for 45 sec, and 72°C for 50 sec, followed by a final extension at 72°C for 7 min. The PCR Max product was subcloned into the *EcoRV* site of pBluescript II SK +/- (Stratagene) to form plasmid pBSMAX6. The identity of this PCR clone to the clone that was isolated by Blackwood and Eisenman (16) was verified by sequencing. This clone contains the full-length coding region of the human Max gene including the insert of 9 amino acids in the N terminus (16).

RNA Analysis. Total RNA was collected from cells by the guanidinium isothiocyanate method, followed by centrifugation through a CsCl cushion (19). RNA Northern blot analysis was performed as described (20). The Northern blot was hybridized to a human Max cDNA probe [labeled by a Multiprime (Amersham) random priming kit] overnight in conditions as described (20). The blot was washed twice in 2× standard saline citrate (SSC)/0.1% SDS at 42°C and twice in 0.2× SSC/0.1% SDS at 60°C before autoradiography. Induction was measured by densitometric analysis (Image Version 1.15) of a screen image generated by a Microtek color/gray scanner.

For RNase protection analysis, RNA (20 µg of total cellular RNA plus RNA probe) was denatured at 80°C for 7 min and hybridized to Max, Myc, and β_2 -microglobulin probes at 56°C for 12–14 h. The Max probe was transcribed by T7 RNA polymerase from pBSMAX6 that was linearized by digestion with *Sma* I. The probe contains 317 nucleotides (nt) from the C terminus of the Max coding region between nt 152 and 468 of the published sequence (16). The Myc probe contains 600 nt from the C terminus region of human Myc coding region and was transcribed by SP6 RNA polymerase from a linearized pGEMMYC plasmid. The Myc probe that encompasses the two human Myc promoters, P1 and P2, was as described (21). The β_2 -microglobulin probe was transcribed from a pSP65 plasmid containing a 145-nt fragment from the 5' end of the human β_2 -microglobulin cDNA (22). RNase treatment was performed as described (21). RNase-resistant fragments were analyzed by electrophoresis in a 5% polyacrylamide sequencing gel.

Fluorescence *in Situ* Chromosomal Hybridization. Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Max probes 6.4 and 6.8 contain 18-kilobase (kb) and 14-kb genomic DNA inserts, respectively, and were isolated after screening of λ EMBL3 random human genomic library with the Max cDNA clone. The procedure used for fluorescence *in situ* hybridization is a modification (23) of the method described by Lichter *et al.* (24). Biotin-labeled probes were prepared by nick-translation using biotin-conjugated dUTP, Bio-11-dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4',6-diamidino-2-phenylindole dihydrochloride.

RESULTS

Max Encodes a Major 2.0-kb mRNA and a Minor 1.7-kb mRNA. The expression of Max RNA was first analyzed in cell lines from various tissues of human or murine origin. A partial Max cDNA, encompassing the full-length coding region of the gene, was obtained by using the PCR, a human cDNA library, and primers flanking the open reading frame reported by Blackwood and Eisenman (16). The partial cDNA obtained was radiolabeled and used for Northern blot analysis of total RNA isolated from primary normal human

skin fibroblasts, the human B-cell lines BJAB and RPMI 1788, mouse BALB/c 3T3 cells, and WEHI-231 cells, a mouse lymphoid tumor cell line. As shown in Fig. 1, we observed a major RNA species of 2.0 kb and a minor species of 1.7 kb that hybridized specifically to Max cDNA. Max mRNA was expressed in all cell lines tested and the sizes of the murine and the human Max RNAs were identical. Both mRNA species of 2.0 kb and 1.7 kb were also observed previously in mouse NIH 3T3 cells (17). The origin of the minor 1.7-kb mRNA is not yet clear, but it is very likely that the 2.0-kb mRNA encodes the full-length Max protein as reported (16, 17). The 1.7-kb mRNA may represent a product of alternative splicing and may encode a Max protein with a deletion of 9 amino acids in the N terminus portion of the protein (16, 17).

Max RNA Is Constitutively Expressed as Myc RNA Levels Are Altered. Max is a specific heterodimerization partner of Myc and enhances the DNA binding activity of Myc to its cognate specific DNA binding site (16, 17). Since Myc expression is tightly linked to cell proliferation, we were interested in studying the expression of Max in various settings in which Myc expression is regulated.

To examine the regulation of Max RNA expression and to compare its level of expression to that of Myc, we performed Northern blot analysis and quantitative RNase protection analysis of Myc and Max RNAs. For the RNase protection assays, a fragment that protects 317 nt of the human Max RNA was used. For the analysis of Myc RNA, a fragment that contains the human Myc first exon or a 600-base-pair fragment of human Myc cDNA was used.

To study the regulation of Max expression after serum stimulation of quiescent fibroblasts, we used mouse BALB/c 3T3 fibroblasts in which Myc RNA level is significantly induced after serum stimulation (3). Myc and Max RNAs were analyzed in quiescent or serum-stimulated cells. The RNA level was determined by Northern blot analysis (Fig. 2) and by RNase protection (data not shown). We observed that Myc RNA was transiently induced after serum addition reaching an $\approx$ 40-fold induction at 3 h and decreasing at 15 h after addition of serum (Fig. 2A). Unlike Myc RNA, Max RNA was expressed in quiescent cells and only an $\approx$ 3-fold induction (as measured by densitometric analysis) of both Max RNAs at 2.0 kb and 1.7 kb occurred 3 h after addition of serum. This induction remained at the same level 15 h after serum addition (Fig. 2B). A similar induction of Max RNA

FIG. 1. Analysis of Max RNA in human and murine fibroblasts and B-cell lines on Northern blots of total cellular RNA from human and murine cell lines. RNA (20 µg) from each cell line was subjected to RNA blot analysis and probed with 32 P-labeled human Max cDNA. The positions of 28S and 18S rRNA and of Max RNAs (2 kb and 1.7 kb) are indicated. HSF, human skin fibroblasts.

was observed (17) after serum stimulation of quiescent NIH 3T3 cells.

Myc RNA level declines when human myeloid leukemia cells, HL60, are induced to differentiate (6, 25, 26). To study the regulation of Max expression during differentiation of HL60 cells, the cells were induced to differentiate with 1.5% DMSO. RNA was extracted from the cells at various times after addition of DMSO and analyzed by quantitative RNase protection (Fig. 3). In agreement with previous reports (6, 26), a significant decline in Myc RNA level was observed 12 h after addition of DMSO (Fig. 3B, lane 3), and Myc RNA was almost undetectable after 72 h (Fig. 3B, lane 5). Max RNA, however, was constitutively expressed during the differentiation of HL60 cells (Fig. 3A), and only a slight decrease in Max RNA level was observed at late time points after

induction of differentiation (Fig. 3A, lane 5). Once again, we observed that Max RNA expression was not tightly linked to cell proliferation and was constitutively expressed in growth-arrested and differentiated cells when Myc RNA level declined.

Myc expression is deregulated in Burkitt lymphoma cells as a consequence of the translocation of one Myc allele to one of the immunoglobulin genes (for reviews, see refs. 1, 2, and 9). Deregulation of Myc expression in Burkitt lymphoma cells was observed by constitutive expression and elevated levels of Myc RNA (27–29). To determine whether a parallel increase in Max RNA level can be observed in Burkitt lymphoma cells, we analyzed the Myc and Max RNA levels in these cells. The RNA was analyzed by a quantitative RNase protection assay in which probes for Myc and Max with the same specific activity were used; the relative abundance of the length of the protected fragment and the number of labeled uridines in each fragment. In several Burkitt lymphoma cell lines that we tested (ST486, PA682, EB-2, and RAMOS) and in BJAB, a human B-cell line, the level of Myc RNA was greater than or comparable to that of Max (Fig. 4, lanes 4–7). In the human B-cell line RPMI 1788, however, the level of Max RNA was greater than that of Myc (Fig. 4, lane 3). The Burkitt lymphoma cells are in a developmental stage in which the normal Myc allele is silent; we observed, however, that Max is expressed in these cells. Since we have

observed that Max RNA expression was not tightly linked to cell proliferation and was constitutively expressed in growth-arrested and differentiated cells when Myc RNA level declined.

FIG. 5. Stability of Myc and Max transcripts. (A) HL60 cells were treated with actinomycin D ($5 \mu\text{g/ml}$) for the following times. Lanes: 1, 0 h; 2, 0.5 h; 3, 1 h; 4, 3 h. Lane 5 contains size markers. Total cellular RNA ($20 \mu\text{g}$) from each time point was analyzed by RNase protection as described in Fig. 4. (B) Transcript survival relative to time zero was determined by liquid scintillation counting of excised bands. The lines of best fit were determined by least-squares analysis, omitting the 0-h point to account for the delay in effect of actinomycin D. Triangles, Max survival; circles, Myc survival. The Max half-life is 3.6 h; the Myc half-life is 45 min.

no evidence that the Max gene is rearranged or translocated in Burkitt lymphoma, we assume that it is actively transcribed in this developmental stage of B cells as it is in quiescent BALB/c 3T3 cells (Fig. 2) and in differentiating HL60 cells (Fig. 3).

Max RNA Is Stable. Using RNase protection assays, we measured the half-life of Max and Myc RNA collected from HL60 cells at various times after treatment with actinomycin D. As shown in Fig. 5A, the stability of the Max RNA was ≈ 5 times greater than that of Myc RNA. Calculations from

computer-generated curves (Fig. 5B) indicated a Max RNA half-life of ≈ 3.6 h and a Myc RNA half-life of 45 min. A similar value for the half-life of Max RNA was obtained with RNA isolated from BJAB cells (data not shown). Our value for the half-life of Myc RNA is slightly greater than that reported (30–32); we believe that this may be due to the amplification of Myc in HL60 cells that delays immediate inhibition of Myc transcription by actinomycin D. The half-life of Max RNA is comparable to the average half-life of most mRNAs in mammalian cells (33). Max mRNA, therefore, does not exhibit the characteristic short RNA half-life of most immediate early genes (34).

Chromosomal Localization of the Max Gene. Deregulation of Myc expression in B-cell (Burkitt lymphoma) and T-cell lymphomas is associated with rearrangement and translocations involving one of the Myc alleles (9, 35). To explore the possibility that lesions in the Max gene are also associated with the development of neoplasia, we identified the chromosomal location of Max.

To localize the Max gene, we performed fluorescence *in situ* hybridization of a biotin-labeled Max probe to normal metaphase chromosomes. Fluorescent signals from biotin-labeled probes were visualized as discrete green-yellow dots on unstained chromosomes; a specific signal was frequently observed on all four chromatids. Hybridization of both of the Max probes simultaneously resulted in specific labeling only of chromosome 14 (Fig. 6). Specific labeling of 14q23 was observed on one (1 cell), two (5 cells), three (11 cells), or all four (8 cells) chromatids of the chromosome 14 homologues in the 25 cells examined. Similar results were obtained in two additional hybridization experiments using these probes. In hybridizations using only a single probe (either 6.4 or 6.8), signal was also observed at 14q23; however, the signal was weaker than in those experiments in which both probes were hybridized simultaneously. Thus, the Max gene is localized to chromosome 14, band q23.

DISCUSSION

Our results demonstrate the presence of a relatively stable Max RNA that is constitutively expressed throughout both induction of cell division and cellular differentiation. Max RNA is less abundant than Myc RNA except in cells that are not rapidly dividing, such as differentiating HL60 cells or quiescent fibroblasts. A modest induction of expression is seen after addition of serum to quiescent cells, and a slight decrease of steady-state levels is observed during HL60 differentiation, but, compared to levels of Myc RNA, Max RNA levels are not significantly altered during these processes. Although Max RNA can be modestly induced by

FIG. 6. *In situ* hybridization of a biotin-labeled Max probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. (a) Counterstained with 4',6-diamidino-2-phenylindole dihydrochloride. (b) Detection of the probe with fluorescein isothiocyanate-conjugated avidin. The chromosome 14 homologues are identified with arrows; specific labeling was observed at 14q23. (c) Partial karyotype of a chromosome 14 homologue illustrating specific labeling at 14q23 (arrowhead).

serum, it does not display the characteristic short half-life of RNAs encoded by most immediate early genes, and, unlike the RNAs of the immediate early genes, its induction is dependent on protein synthesis (17, 34). The requirement of protein synthesis for the induction of Max after serum stimulation raised the possibility that this induction is regulated by immediate early gene products and may be regulated by Myc itself (17). The expression of Max RNA, however, cannot be totally dependent on the products of the immediate early genes (including Myc) since the induced level is retained even 15 h after serum stimulation and Max RNA is expressed at relatively high levels in physiological conditions in which the immediate early genes are not expressed. The constitutive expression of Max suggests that overexpression of Max in the cells will not induce transformation. Indeed, unlike Myc (36), overexpression of Max in Rat-1a cells does not elicit morphological transformation of these cells (unpublished results).

The inverse levels of Max and Myc expression in growth-arrested cells vs. proliferating cells suggest that Max may modulate Myc function in an antagonistic fashion (see also ref. 18). For example, relatively high levels of Max in nonproliferating cells may compete with Myc for binding to the same DNA binding site, thus inhibiting Myc activity. In proliferating cells and in tumor cells in which Myc is deregulated, relatively high levels of Myc may enable it to bind its cognate DNA binding site either as a heterodimer with Max or as a homodimer.

The stability of Max RNA is approximately equal to the stability of most poly(A)⁺ RNA transcripts (33). The turnover of this transcript is insufficient to account for modulation of Myc activity unless the degradation of Max RNA is somehow regulated. The long 3' noncoding region seen in the murine cDNA (17) may serve to govern its stability or as a template for a regulated nucleolytic activity that can degrade Max RNA after an appropriate stimulus. In addition, it is possible that, although Max RNA is expressed constitutively, the translation of this RNA is regulated. The turnover of Max protein has not been determined in the present studies but it may also be subject to regulation.

Another, and perhaps more likely, stage of regulation of Max activity may be at the posttranslational level. The C terminus of Max contains several consensus target sites for casein kinase II phosphorylation and several serine/threonine residues that can be target for phosphorylation by other kinases. Deletion of this C-terminal portion of Max led to enhanced DNA binding both as a dimer with Myc and alone, as a homodimer (ref. 17; C. Amin and N.H., unpublished results). Thus, phosphorylation of Max could regulate its DNA binding abilities and its dimerization with Myc. This would provide a mechanism for rapid translation-independent control of Max activity and, therefore, could potentially modulate Myc activity.

By using fluorescence *in situ* hybridization, we have localized the Max gene to chromosome 14, band q23; this locus is proximal to the location of the Fos and transforming growth factor β_3 genes (14q24) (ref. 37). With respect to the recurring abnormalities in human tumors, this region of chromosome 14 is involved in deletions in B-cell chronic lymphocytic leukemia and malignant lymphomas [del(14)(q22-q24)] and in the t(12;14)(q13-15;q23-24) in uterine leiomyomas (38). Whether the function or the regulation of the Max gene is altered as a result of these rearrangements is unknown.

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