Testis-specific expression of the human MYCL2 gene

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Received December 14, 1990; Revised and Accepted April 16, 1991

ABSTRACT

We have characterized the expression of MYCL2, an intronless X-linked gene related to MYCL1. RNase protection analysis of a panel of human normal and tumor tissues has revealed that MYCL2 is expressed almost exclusively in human adult normal testis; much lower levels of transcript were detected in one human lung adenocarcinoma. No MYCL2 transcript was found in human testis RNA obtained from second trimester fetuses. This observation suggests a germ cell rather than somatic cell origin of the transcript and possible developmental regulation of MYCL2. Northern blot analysis of poly(A)$^+$ RNA from adult human normal testis with an antisense riboprobe revealed a transcript of approximately 4.8-kb, which is in agreement with the size predicted from the MYCL2 nucleotide sequence. Antisense transcripts were found spanning regions of MYCL2 corresponding to all three exons of MYCL1. No sizable open reading frame was seen for the MYCL2 antisense transcripts suggesting that they may represent either regulatory sequences or an intron of a gene encoded by the complementary strand. RNase protection assays and the 5′ RACE protocol (Rapid Amplification of cDNA Ends) were used to address the localization of the transcription start site of the MYCL2 sense transcript and different putative promotors and transcription regulatory elements have been identified.

INTRODUCTION

The myc family of proto-oncogenes consists of several members including MYC, MYCN, MYCL1, MYCL2, B-myc, and s-myc[1]. These genes have regions of sequence identity and some are known to have structural and functional similarities such as nuclear localization, amplification and/or overexpression in various tumors, cooperation with activated HRAS to yield malignant transformation of primary rat embryo fibroblasts, and encoding DNA-binding phosphoproteins (3,4). However, members of the myc gene family show different temporal and tissue-specific patterns of expression and distinct mechanisms of transcriptional regulation in various normal and tumor tissues, implying differences in their respective functions.

MYCL2 is an intronless X-linked gene with a substantial degree of nucleotide sequence identity to the three exons of MYCL1 including conservation of splice junction consensus sequences (5,6). The finding of an open reading frame of 1194-bp in MYCL2 suggested that this intronless gene might be functional (6). Another recently characterized intronless member of the myc family of proto-oncogenes, s-myc, expressed in small amounts in various rat organs, was shown to be transcribed and translated *in vitro* and is believed to have suppressive effects on tumorigenicity (7). These two genes add to a growing list of other functional intronless genes in eukaryotes.

We undertook this investigation to address function of MYCL2. In an initial search for MYCL2 expression (6), we did not detect a transcript, although we were limited to obtaining RNA from a selected number of tissues at certain cell cycle and/or developmental stages. Because the regulated expression of several proto-oncogenes, including other myc family members, has been established in germ cells (8–15), we decided to explore possible MYCL2 expression in gonadal tissues. We have concentrated our study on expression of MYCL2 in human testis because of the difficulty in obtaining young adult human normal ovaries and the small germ cell to stromal cell ratio in this organ. Several genes, including proto-oncogenes, enzymes, histones, and structural genes, are thought to be transcribed in the testis either exclusively, in alternate unique forms, or in increased amounts (13). Gonads are the only normal adult tissues in which Int-1 (12) and the intronless Mos proto-oncogene (11) are expressed in significant amounts. This precedent of gonad-specific expression of these proto-oncogenes further prompted us to look for MYCL2 expression in the testis. Herein we report *in vivo* expression of MYCL2 and characterize the MYCL2 transcript in human adult testis by RNase protection, Northern blot, and cDNA analyses to gain insight into the nature of the message, the transcription start site, and possible promoter and transcription regulatory elements.

MATERIALS AND METHODS

RNA Isolation

Total cellular RNAs were extracted (16) from human tumor cell lines and from various human fetal and adult normal tissues and human tumor tissues obtained in accordance with guidelines established by our institutional review board. Poly(A$^+$) RNA was isolated (17) from human placenta and from histologically normal human adult testes obtained from therapeutic

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orchiectomies of prostatic cancer patients without prior history of hormonal or chemotherapy. The human adult normal testis tissue did not contain tumor or any other intrinsic lesion, and showed minimal or no atrophy. Greater than 80% of seminiferous tubules were intact and contained germinal epithelium, and greater than 50% of seminiferous tubules were found to contain mature sperm (i.e., evidence for mature spematogenesis).

Northern Blot Analysis

RNAs were electrophoresed in denaturing agarose-formaldehyde gels and transferred to GeneScreen (Dupont, Wilmington, DE) filters (18). Filters were prehybridized at 55°C in 5×SSC (1×SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.05 M sodium phosphate (pH 7), 0.5% SDS, 5×Denhardt's solution (1×Denhardt's is 2% Ficoll, 2% polyvinylpolypyrrolidone, and 2% bovine serum albumin), 0.05 mg/ml sonicated herring sperm DNA, 0.5 mg/ml yeast tRNA, and 50% formamide. Hybridization of filters was performed at 55°C in 10% dextran sulfate, 5×SSC, 0.02 M sodium phosphate (pH 7), 1×Denhardt's solution, 0.05 mg/ml sonicated herring sperm DNA, 0.5 mg/ml yeast tRNA, and 50% formamide using either the MYCL2 190-base *Sst*I-*Bam*HI (Fig. 3A) or the MYCL1 1.5-kb *Hin*dIII-*Eco*RI (Fig. 3B) ^{32}P-labelled antisense riboprobe synthesized by the pGEM-3 system (Promega, Madison, WI). Filters were washed in 0.1×SSC in 0.1% SDS at 55°C and autoradiographed for 3 to 10 days using XAR-5 film (Kodak, Rochester, NY) with intensifying screens at −70°C.

MYCL2 fragments were subcloned from a MYCL2 clone obtained from *Eco*RI-digested, size-fractionated human T cell DNA cloned into λ gt WES (6). MYCL1 fragments were subcloned from a MYCL1 clone obtained from *Eco*RI-digested, size-fractionated human T cell DNA cloned into λ gt 10 (19). 'Exons' in the intronless MYCL2 gene were defined on the basis of corresponding exonic regions in MYCL1.

RNase Protection Analysis

To characterize expression of MYCL2, RNase protection analysis (20) of 20−25 ug of total cellular RNAs was performed using the following ^{32}P-labelled riboprobes derived from both strands of MYCL2 fragments subcloned into pGEM-3: 190-bp *Sst*I-*Bam*HI (Fig. 1A), 680-bp *Hin*dIII-*Kpn*I (Fig. 1B), and 545-bp *Hin*cII-*Hin*dIII (Fig. 5). To assess protection between the homologous MYCL1 and MYCL2 genes, RNase protection analyses of synthetic *Eco*RI-*Stu*I MYCL1 RNA with MYCL2 190-base *Bam*HI-*Sst*I antisense riboprobe (Fig. 2A) and of synthetic *Sma*I-*Pvu*II MYCL1 RNA with MYCL2 680-base

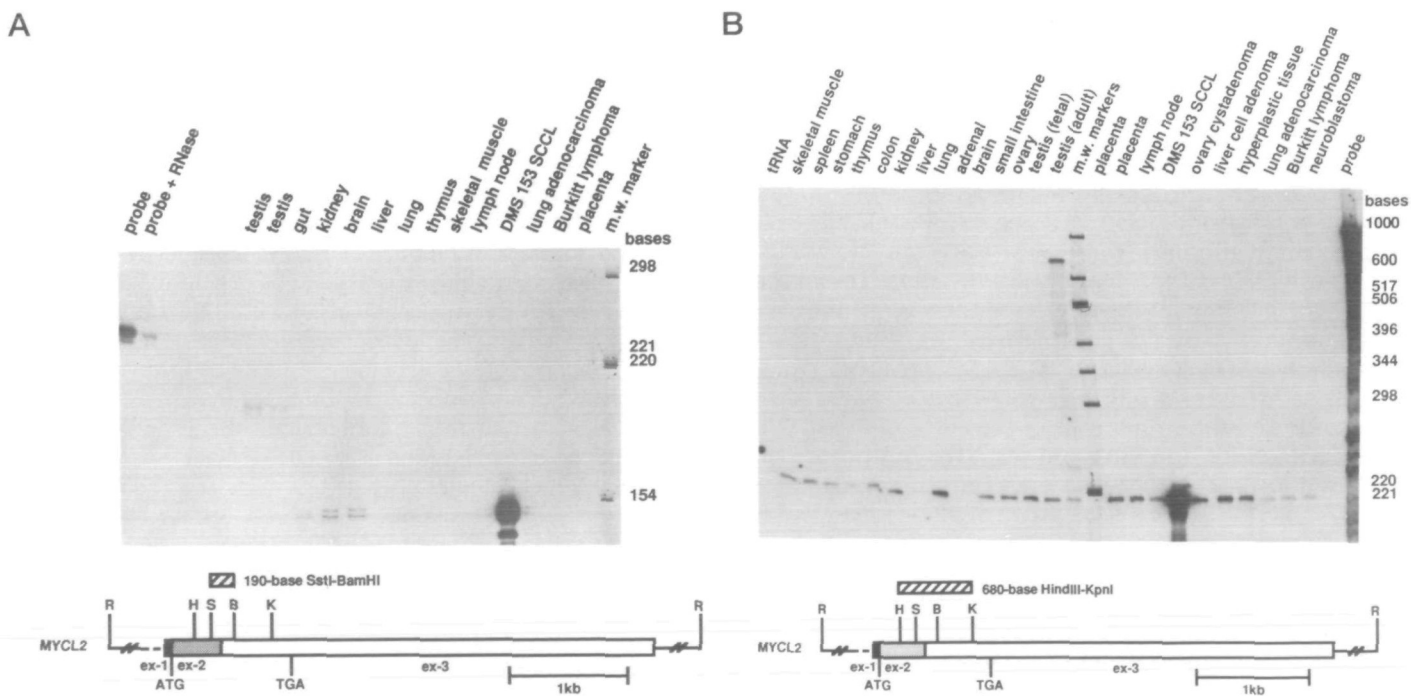

Fig. 1. (A) Autoradiograph of RNase protection assay of a panel of total RNAs obtained from human normal and tumor tissues using the MYCL2 *Sst*I-*Bam*HI antisense riboprobe (1 day exposure). All normal RNAs were obtained from fetal tissues, except for two adult testis samples. Tumor RNAs were obtained from adult tumors or cell lines. MYCL2 transcript, represented by a fully protected 190-base hybridizing band, is seen only in RNA from adult testis. Several protected bands of lower molecular weight prominent in the DMS 153 SCCL (Small Cell Carcinoma of the Lung) cell line may represent partially protected bands from cross hybridization with MYCL1 RNA which is overexpressed in this cell line. A subset of these bands is also seen at lesser intensity in RNAs obtained from other tissues and likely represents MYCL1 cross hybridization. **(B)** Autoradiograph of RNase protection assay of a larger panel of human normal and tumor tissue total RNAs using the MYCL2 *Hin*dIII-*Kpn*I antisense riboprobe (10 day exposure). All normal RNAs were obtained from fetal tissues, except for one adult testis sample. Tumor RNAs were obtained from adult tissues or cell lines. MYCL2 transcript, represented by a fully protected 680-base hybridizing band, is seen in RNAs from adult testis and in a much lesser amount from a lung adenocarcinoma. No MYCL2 transcript is detected in RNA from fetal testis. Numerous hybridizing bands of lower molecular weight prominent in the DMS 153 SCCL cell line may represent partially protected bands from cross hybridization with the overexpressed MYCL1 RNA. A band at approximately 220-bases is also seen in RNA obtained from other tissues and likely represents MYCL1 cross hybridization. Molecular weight markers are DNA fragments generated by digestion of pBR322 with *Eco*RI and *Hin*fI. 'Exons' in the intronless MYCL2 gene are indicated with reference to exons in MYCL1. The dashed line shown on the MYCL2 restriction map indicates the approximate transcription start site. Restriction sites indicated are **R**=*Eco*RI, **H**=*Hin*dIII, **S**=*Sst*I, **B**=*Bam*HI, and **K**=*Kpn*I.

*Hin*dIII-*Kpn*I antisense riboprobe (Fig. 2B) were performed. Gels were dried and autoradiographed for 1 to 14 days using XAR-5 film with intensifying screens at −70°C.

5′ RACE (Rapid Amplification of cDNA Ends)

Reverse Transcription

Ten ug of total cellular RNA from normal human adult testis was treated with 60 units of DNase I (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at 37°C to remove any genomic DNA contamination. DNase was heat inactivated and removed by phenol and chloroform extractions. Following precipitation, RNA was heated for 3 min at 65°C and quenched on ice. Reverse transcription of this RNA was performed in a 10 ul total reaction volume, using 20 pmoles of a gene-specific MYCL2 reverse transcription primer, RT, (TTCTTTACTGTCACGTCG), 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 25 units of RNase-inhibitor (Boehringer Mannheim, Indianapolis, IN), 0.2 mM of each dNTP in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, and 10 mM dithiothreitol, for 1hr at 42°C.

Tailing and Amplification

The RACE protocol (21) was performed as follows: RT primer was removed from the cDNA product using a Centricon-100 microconcentrator (Amicon Division, Danvers, CT) as specified by the manufacturer. The cDNA pool was tailed on the 5′ end with deoxyadenosine (Fig. 6) using 10 units of terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) and supplied buffer as directed by the manufacturer. Approximately 2% of the tailed cDNA was amplified by PCR (polymerase chain reaction) (22) using 10 pmoles of ***ADT_{17} primer consisting of an adaptor portion with the restriction sites *Eco*RI, *Bam*HI, and *Xba*I attached to 17 d(T) residues (CGAATTCGGATCC-$TCTAGAT_{17}$), 25 pmoles of adaptor primer, ***AD, containing the above three restriction sites and no T_{17}, and 25 pmoles of a MYCL2-specific primer, designated AMP1 primer (AAGCAGGTCACTCACCG). Amplification was carried out in a 50 ul reaction volume containing PCR buffer (10 mM Tris (pH 8.4), 50 mM KCl, 1 ug gelatin, 1.5 mM $MgCl_2$) and 0.2 mM of each dNTP topped with 40 ul of mineral oil. Following denaturation at 95°C for 5 min and cooling to 72°C, 1.25 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) was added to each reaction, and 1 cycle of annealing at 50°C for 5 min and extension at 72°C was performed. Forty cycles of denaturation at 94°C for 40 sec, annealing at 50°C for 1 min, extension at 72°C for 3 min, and a final extension at 72°C for 15 min were carried out with a DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT). For added specificity, 2 ul (4%) of the product generated was amplified in the same manner as above, using another MYCL2 gene-specific nested 3′ primer, AMP2 (AGCTTTCCAGTA GTCCTG), with ***ADT_{17} and ***AD 5′ primers.

Southern Blot Analysis, cDNA Cloning, and Nucleotide Sequencing

The final product of the RACE protocol was digested with *Xba*I (restriction site present in adaptor primer and incorporated into the 5′ end of cDNAs) and *Taq*I (restriction site present in amplified portion of MYCL2 sequence) (Fig. 6). The digested cDNAs were electrophoresed in a 1.5% agarose gel and transferred to a nitrocellulose filter as described (23). The filter was prewashed at 65°C for 1 hr in 3×SSC and 0.1% SDS, and prehybridized at 37°C for 1 hr in 6×SSC, 5×Denhardt's solution, 0.05% sodium pyrophosphate, 0.1 mg/ml sonicated salmon sperm DNA, and 0.5% SDS. Hybridization was performed at 42°C overnight in 6×SSC, 5×Denhardt's solution, 0.05% sodium pyrophosphate, and 0.1 mg/ml yeast tRNA using an end-labelled oligonucleotide (32-bases) probe derived from the second exon of MYC, GAGGATATCTGGAAGAAATTCG-AGCTGCTGCC. The filter was washed at room temperature in 6X SSC and 0.05% sodium pyrophosphate and autoradiographed using XAR-5 film with intensifying screens at −70°C. Hybridizing DNA fragments, most likely representing different species of MYCL2-specific cDNAs and ranging in size from 50-bp to 230-bp, were gel eluted and subcloned into Bluescript vector (Stratagene, La Jolla, CA). Subclones were further selected for MYCL2 specificity by digestion with restriction enzymes *Sst*I and *Xho*I (sites present in Bluescript polylinker flanking insert), electrophoresed, blotted, and hybridized as described above to either the end-labelled

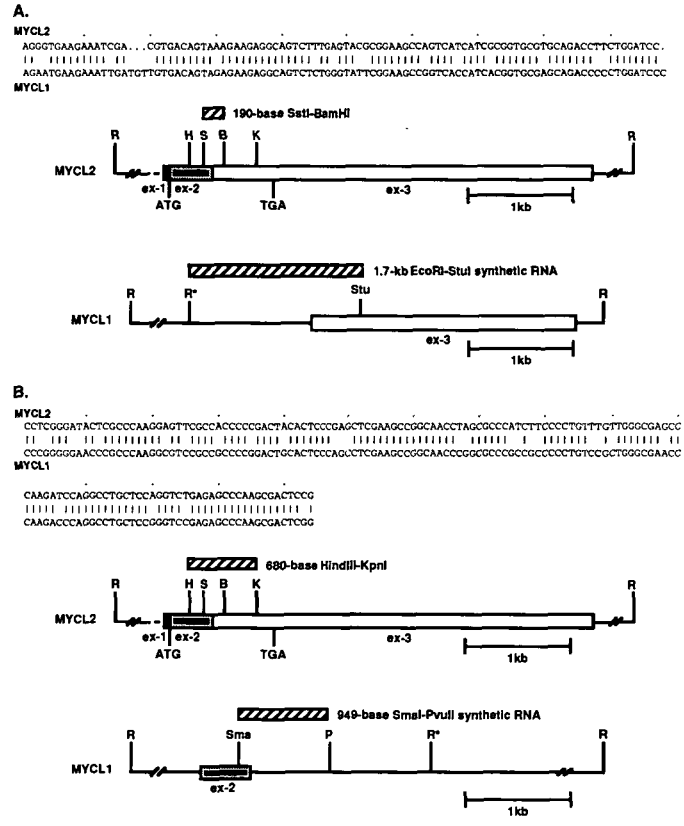

Fig. 2. Sequence identity between **A)** MYCL2 *Sst*I-*Bam*HI and MYCL1 *Eco*RI-*Stu*I DNA fragments and **B)** MYCL2 *Hin*dIII-*Kpn*I and MYCL1 *Sma*I-*Pvu*II fragments (6,26). To assess cross hybridization of MYCL1 to MYCL2 riboprobes constructed for RNase protection experiments shown in Fig. 3, RNase protection assays of the following were performed: synthetic sense RNA was generated from **A)** the 1.7-kb MYCL1 *Eco*RI-*Stu*I fragment and hybridized with the MYCL2 *Sst*I-*Bam*HI antisense riboprobe or **B)** the 949-bp MYCL1 *Sma*I-*Pvu*II fragment and hybridized to the MYCL2 *Hin*dIII-*Kpn*I antisense riboprobe. No fully protected hybridizing bands were detected (data not shown). These results support the usefulness of the MYCL2 riboprobes in their specificity of detection of MYCL2 message by RNase protection assay, despite a substantial degree of sequence identity between the two genes. The dashed line shown on the MYCL2 restriction map indicates the approximate transcription start site. Restriction enzymes indicated are **H**=*Hin*dIII, **S**=*Sst*I, **B**=*Bam*HI, **K**=*Kpn*I, **Stu**=*Stu*I, **Sma**=*Sma*I, **P**=*Pvu*II **R**=*Eco*RI, and **R***=polymorphic *Eco*RI (MYCL1 only).

oligonucleotide (32-bases) from the second exon of MYC or an end-labelled oligonucleotide (20-bases) derived from the MYCL2 second exon, AGCAAGCAAGCCAACATGGA, about 100-bp upstream of the *Taq*I restriction site (Fig. 7). The nucleotide sequence of each positive subclone was determined by the dideoxy chain termination method (24) using T7 or T3 promoter primers. University of Wisconsin Genetics Computer Group software (25) was used for sequence analysis.

RESULTS

Detection of MYCL2 Transcript by RNase Protection

To identify a potential MYCL2 transcript, RNase protection assay was chosen due to its sensitivity and specificity. Following hybridization of a panel of second trimester human fetal normal tissue total RNAs, human adult normal testis RNA, and tumor tissue and cell line RNAs with the MYCL2 *Sst*I-*Bam*HI antisense riboprobe, a 190-base protected band was seen only in the adult testis RNA sample (Fig. 1A); this size is in agreement with the size predicted for full protection. Subsequently, a larger panel of total RNAs, including fetal testis and ovary, as well as additional tumor tissue RNAs was tested by RNase protection with a MYCL2 *Hin*dIII-*Kpn*I antisense riboprobe, encompassing a larger area of exons 2 and 3 (Fig. 1B). Again, a fully protected hybridizing band of the predicted size (680-bases), representing MYCL2 message was seen in normal adult testis total RNA. Interestingly, no MYCL2 message was detected in RNA from fetal testis tissue. In addition, a much fainter band of 680-bases was detected in total RNA from a lung adenocarcinoma.

Sequence comparison of either the MYCL2 *Sst*I-*Bam*HI or the MYCL2 *Hin*dIII-*Kpn*I probe to corresponding regions of MYCL1 reveals sequence similarities of 85% and 84%, respectively. However, absence of fully protected bands upon hybridization of MYCL2 riboprobe to DMS 153 SCCL (Small Cell Carcinoma of the Lung) cell line RNA, known to overexpress MYCL1 (Fig. 1A, 1B) provides an internal negative control for cross hybridization at the fully protected size in each experiment. In addition, we confirmed the specificity of our MYCL2 riboprobes in detection of MYCL2 message by hybridizing MYCL2 probes to synthetic RNAs derived from MYCL1 DNA fragments. Nucleotide sequence identity and restriction maps of the constructs used are shown in Fig. 2. Hybridization of the MYCL2 *Sst*I-*Bam*HI antisense riboprobe to MYCL1 *Eco*RI-*Stu*I synthetic RNA and of the MYCL2 *Hin*dIII-*Kpn*I antisense riboprobe to MYCL1 *Sma*I-*Pvu*II synthetic RNA did not detect any fully protected hybridizing bands (data not shown). These results confirm specificity of MYCL2 riboprobes in detection of MYCL2 message in this assay.

Of note is the presence of several bands smaller than the expected fully protected MYCL2 message in the RNase protection assays. In Fig. 1A, bands of about 150-bases and, in Fig. 1B,

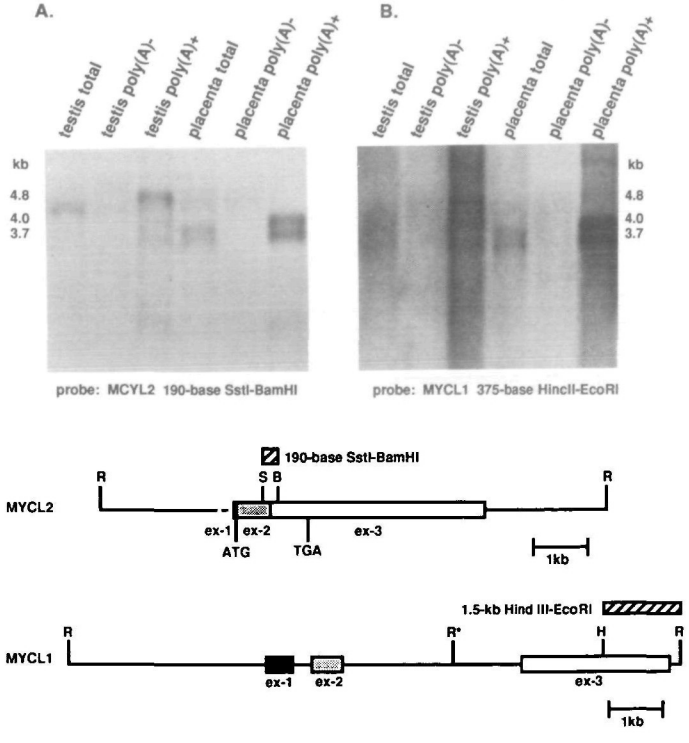

Fig. 3. Autoradiograph of Northern blots of total and poly(A)+ RNAs extracted from normal human adult testis and placenta and hybridized with **(A)** MYCL2 *Sst*I-*Bam*HI antisense riboprobe (10 day exposure) or **(B)** MYCL1 *Hin*dIII-*Eco*RI antisense riboprobe (3 day exposure). In **(A)** a MYCL2-specific 4.8-kb hybridizing band is present in the testis poly (A)+ and the testis total RNAs. (The slight difference in apparent size of bands in 20 ug of total vs. 3 ug of poly (A)+ RNAs may be an artifact of migration due to different amounts of RNA in each lane). Cross hybridization is seen to 4.0-kb and 3.7-kb MYCL1 messages in total and poly (A)+ RNAs from placenta. In **(B)** MYCL1-specific hybridizing bands of 4.0-kb and 3.7-kb are detected in poly (A)+ and total RNA from placenta. The dashed line shown on the MYCL2 restriction map indicates the approximate transcription start site. Restriction enzymes indicated are **S**=*Sst*I, **B**=*Bam*HI, **H**=*Hin*dIII, **R**=*Eco*RI, and **R***=polymorphic *Eco*RI (MYCL1 only).

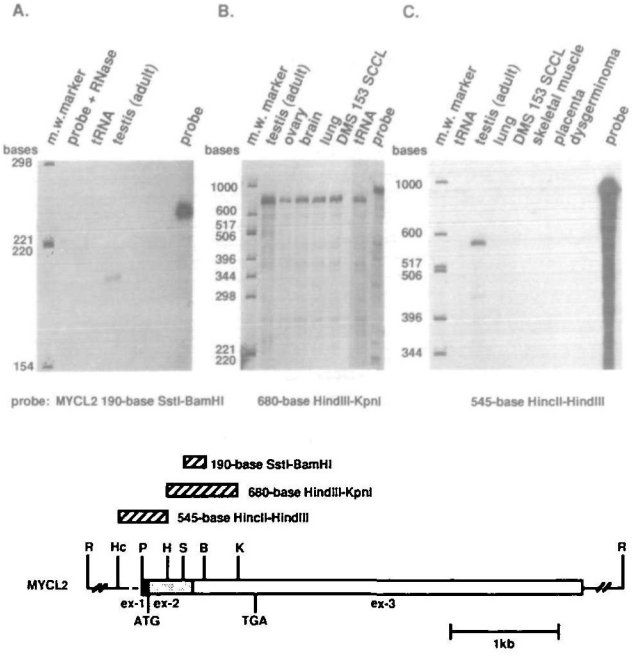

Fig. 4. Autoradiograph of RNase protection assay of a panel of human normal and tumor total RNAs using **A)** the MYCL2 *Sst*I-*Bam*HI sense riboprobe (1 day exposure) **B)** the MYCL2 *Hin*dIII-*Kpn*I sense riboprobe (3 day exposure) or **C)** the MYCL2 *Hin*cII-*Hin*dIII sense riboprobe (2 week exposure). All RNAs were derived from human fetal tissues except for RNAs from human adult testis and a dysgerminoma, and from the DMS 153 SCCL (Small Cell Carcinoma of the Lung) cell line. In each assay, a protected band of the predicted size is seen in testis RNA: **A)**190-bases, **B)**680-bases, and **C)**545-bases. These bands represent antisense RNA derived from the corresponding portion of the MYCL2 locus. Restriction enzymes indicated are **R**=*Eco*RI, **Hc**=*Hin*cII, **P**=*Pvu*II, **H**=*Hin*dIII, **S**=*Sst*I, **B**=*Bam*HI, and **K**=*Kpn*I.

a hybridizing band of about 220-bases are present in several RNA samples. These bands are particularly intense in the RNA sample from the DMS 153 cell line, which is known to overexpress MYCL1 (6). It is likely that these lower molecular weight bands are the result of cross hybridization of MYCL2 riboprobes to MYCL1 message and protection in regions of sequence identity between the two genes.

Northern Blot Analysis of MYCL2 RNA

Northern blots of 20 ug of total and poly(A)$^-$ RNAs, and 3 ug of poly(A)$^+$ RNA from human adult normal testis and placenta were hybridized with the MYCL2 *Sst*I-*Bam*HI antisense riboprobe and a transcript of approximately 4.8-kb was detected in testis poly(A)$^+$ and testis total RNAs (Fig. 3A). The size of the transcript corresponds to that predicted from the MYCL2 nucleotide sequence (5,6). We believe the slight difference in apparent size of bands in total (20 ug) vs. poly (A)+ (3 ug) RNAs is an artifact of migration due to different amounts of RNA in each lane (i.e. the message which co-migrates approximately with the 28S rRNA appears lower in the 20 ug of testis total RNA sample than in the 3 ug of poly (A)+ testis RNA sample).

In addition, there are two hybridizing bands, 4.0-kb and 3.7-kb, in the placenta total and poly(A)$^+$ RNAs. Because sizes of these transcripts correspond to MYCL1 mRNA generated from alternative splicing and polyadenylation site selection (26), we believe these bands represent cross hybridization of MYCL2 probe to MYCL1 RNA. To examine this possibility, we hybridized total, poly(A)$^-$, and poly(A)$^+$ RNAs from normal human adult testis and placenta with a MYCL1 *Hin*dIII-*Eco*RI antisense riboprobe (Fig. 3B). MYCL1 hybridizing bands of 4.0-kb and 3.7-kb were present in placenta poly(A)$^+$ and total RNA confirming cross hybridization of MYCL2 probe to MYCL1 transcripts.

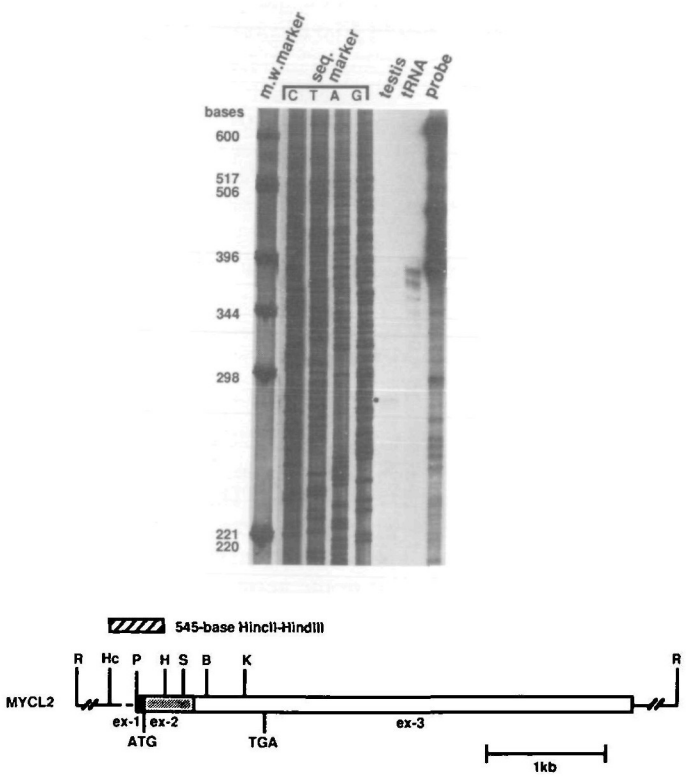

Fig. 5. Autoradiograph of RNase protection assay of human adult testis RNA using the MYCL2 *Hin*cII-*Hin*dIII antisense riboprobe spanning the 5′ border of the gene. The protected fragment in testis RNA (indicated by a black dot to the left of the band) is determined to be approximately 284-bases in size based on the DNA sequence marker run in adjacent lanes. From this data, the transcription start site is estimated to be near a *Pvu*II site about 280-bp upstream of the *Hin*dIII site. Molecular weight markers are DNA fragments generated by digestion of pBR322 with *Eco*RI and *Hin*fI. The dashed line shown on the MYCL2 restriction map indicates the approximate transcription start site. Restriction enzymes indicated are **R**=*Eco*RI, **Hc**=*Hin*cII, **P**=*Pvu*II, **H**=*Hin*dIII, **S**=*Sst*I, **B**=*Bam*HI, and **K**=*Kpn*I.

Presence of MYCL2 Antisense RNA

The use of sense probes as controls in our RNase protection assays led to the detection of MYCL2 antisense RNA. Human adult normal testis total RNA hybridized separately with MYCL2 *Sst*I-*Bam*HI, *Hin*dIII-*Kpn*I, or *Hin*cII-*Hin*dIII sense riboprobes each revealed a fully protected band of the expected size (190-bases, 680-bases, or 545-bases, respectively), representing MYCL2 antisense transcripts (Fig. 4). In the protection assay using the 680-base *Hin*dIII-*Kpn*I probe, the testis RNA sample shows a triplet of bands in the 600 to 800 base range. The bottom band in the triplet represents the protected MYCL2 message in the testis RNA sample. The two upper bands in the triplet appear as a doublet in all other RNA samples, including tRNA hybridized with this probe, and represent background bands due to nonspecific hybridization.

Probes used and antisense messages detected corresponded to regions of MYCL2 ranging from about 260-bp upstream of exon 1 through about 400-bp of exon 3 (Fig. 4). MYCL2 antisense RNA was not seen in other tissues tested. Note the absence of any partially protected specific bands in other tissue RNAs including the DMS 153 SCCL cell line RNA, unlike those seen using antisense riboprobes. This may indicate lack of cross hybridization of MYCL2 sense riboprobes to MYCL1 antisense message or absence of MYCL1 antisense transcript in these tissues.

Characterization of MYCL2 Sense Transcript by RNase Protection and 5′ RACE Analyses

To identify the 5′ boundary of MYCL2 and to rule out the possibility of transcription initiation from an upstream promoter

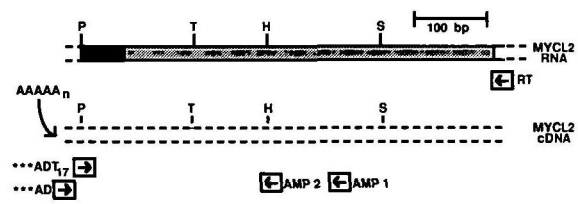

Fig. 6. Schematic representation of the 5′ RACE (Rapid Amplification of cDNA Ends) protocol used to generate MYCL2 cDNA and characterize the transcription start site. A MYCL2-specific reverse transcription primer (RT), located near the predicted 5′ end, was used to generate MYCL2 cDNA from adult testis RNA. After the addition of a poly A tail to the 5′ end of the cDNA, the cDNA was amplified by the polymerase chain reaction, using a 3′ nested gene-specific amplification primer (AMP1) and a 5′ primer (***ADT$_{17}$) consisting of an adaptor portion containing the restriction sites *Eco*RI, *Bam*HI, and *Xba*I attached to 17 d(T) residues: CGAATTCGGATCCTCTAGAT$_{17}$. Subsequent rounds of amplification were performed with AMP1 and adaptor (***AD) primers. For increased specificity, the product generated was then amplified using another nested gene-specific primer (AMP2) and the ***ADT$_{17}$ and ***AD primers. The final product was digested with *Taq*I (restriction site present in amplified portion of MYCL2) and *Xba*I (present in adaptor and part of amplified fragments). cDNAs were subcloned into Bluescript vector for characterization of their 5′ ends by DNA sequencing.

of another gene, we have characterized transcription initiation in MYCL2. Based on nucleotide sequence similarity with MYCL1, we had estimated the 5′ boundary of MYCL2 to lie within the *Hinc*II-*Hin*dII 545-bp fragment (Fig. 5). RNase protection assay of human adult normal testis total RNA using the *Hinc*II-*Hin*dIII antisense riboprobe yielded a specific protected transcript of approximately 284-bases, which indicates transcription initiating from approximately the middle of the *Hinc*II-*Hin*dIII fragment near a *Pvu*II site. Results of four independent RNase protection experiments are summarized in Fig. 7. These data localize the start site of the transcript to the region around the *Pvu*II site; start site of the transcript was calculated from the size of the protected fragment as estimated by the DNA sequencing products electrophoresed in adjacent lanes (Fig. 5). The difference of 3-bases in the estimated sizes of transcripts may reflect the error range in calculation of size by this method or may represent different start sites of the transcript.

To address the possibility of another upstream promoter, intron, or additional exonic sequences, we decided to construct MYCL2 cDNA using the 5′ RACE protocol and to localize the MYCL2 transcription start site by sequencing cDNA clones. The 5′ RACE cloning strategy is outlined in Fig. 6. Adult human normal testis total RNA was pretreated with DNase I to remove any possible genomic DNA contamination. For construction of cDNA, a primer derived from the third exon of MYCL2 was used for reverse transcription. The use of a gene-specific primer located close to the predicted 5′ end increases the likelihood of obtaining full length MYCL2-specific cDNA. Separate rounds of amplification using two nested gene-specific primers also provide additional MYCL2-specificity to the amplified product. Prior to subcloning, amplified cDNAs were digested with *Taq*I restriction enzyme (site present in amplified MYCL2 sequence) and *Xba*I (site present in adapter sequence which is incorporated into the 5′ end of amplified sequences). Electrophoresis of digested products in a 1.5% agarose gel yielded an ethidium bromide-stained lane of DNA fragments, presumably including products of nonspecific reverse transcription and/or amplification. Southern blot analysis of this DNA hybridized with an end-labelled second-exon MYC oligonucleotide having 90% sequence identity to MYCL2 was performed. Only the portion of cDNA products which hybridized to the MYC oligonucleotide were gel eluted and subcloned into Bluescript vector. These subclones were screened by digestion with *Taq*I and *Xba*I to recover the subcloned fragment, as well as by hybridization with either the second-exon MYC oligonucleotide or an oligonucleotide derived from the MYCL2 second exon located about 100-bp upstream of the *Taq*I restriction site (Fig. 6). These steps have provided further selection for MYCL2-specific transcripts. Of 37 clones analyzed, nine were MYCL2 cDNAs. The generation of MYCL2 cDNA from adult human normal testis by the RACE protocol confirms the presence of MYCL2 transcripts in this tissue. Nucleotide sequence analysis of the MYCL2 cDNA has defined the 5′ end of each of the clones (Fig. 7). Of nine sequenced clones which were MYCL2 cDNAs, two were identified in which the 5′ most nucleotide was located about 30-bp downstream of the start site estimated from RNase protection experiments. This size difference may be due to the method of transcript size estimation by RNase protection or it may represent the presence of alternate transcription start sites. Start sites of clones generated by the RACE protocol spanned an area of 80 nucleotides. It is possible that some of the shorter clones are products of premature termination by reverse transcriptase, but the heterogeneity in the number of different 5′ ends may suggest initiation of transcription from multiple sites. Of note, none of these sites extended upstream of the 5′ boundaries as determined by RNase protection. These data make unlikely the possibility of transcription of MYCL2 from a promoter of an upstream gene and support the existence of multiple transcription start sites near, or downstream of, the *Pvu*II site.

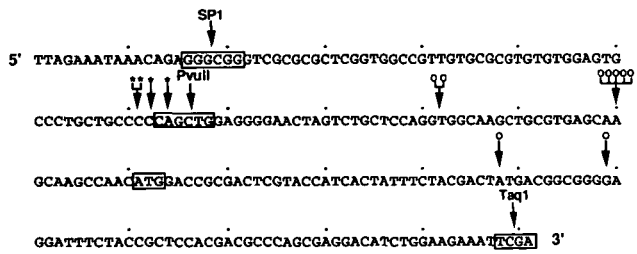

Fig. 7. Linear sequence of the 5′ region of the MYCL2 gene (6) illustrates results of RNase protection and 5′ RACE (Rapid Amplification of cDNA Ends) experiments to determine the MYCL2 transcription start site. Arrows with stars indicate transcription start sites determined in four different RNase protection experiments using MYCL2 *Hinc*II-*Hin*dIII antisense riboprobe hybridized to adult testis RNA. Transcription start sites were determined by calculating the size of protected fragments based on the DNA sequence marker (see Fig. 5). Arrows with open circles represent the most 5′ nucleotide in each of the MYCL2 cDNAs generated by the 5′ RACE protocol. These sites may represent alternate transcription start sites or different points of termination of reverse transcription along the RNA template. Of nine clones analyzed, the 5′ most site seen is about 30-bp downstream of transcription start sites estimated from RNase protection experiments. None of the clones generated by the 5′ RACE protocol extend 5′ of the transcription start site determined by RNase protection. These data help rule out the possibility of transcription of MYCL2 from an upstream promoter of another gene, and support the existence of one or multiple transcription start sites near or downstream of the indicated *Pvu*II site in the MYCL2 sequence. An Sp1 recognition site, which may play a role in activation of MYCL2 transcription from one or several sites, is indicated about 60-bp upstream of this *Pvu*II site.

DISCUSSION

MYCL2 Expression

Our studies have shown expression of MYCL2 in human adult normal testis. MYCL2 transcript was not detected in human normal testis from second trimester fetuses. The lack of detectable transcription in fetal testis, which is thought to possess a much smaller proportion of germ cells as compared to adult testis, could signify expression of MYCL2 in the germ cell rather than the somatic cell compartment of the testis. Alternatively, the temporal

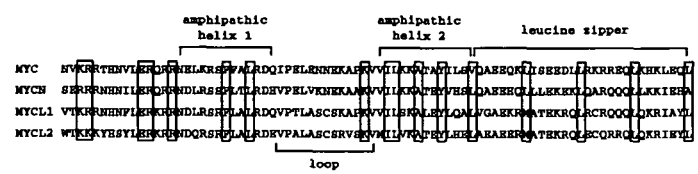

Fig. 8. Comparison of predicted carboxy terminal amino acid sequences of human MYC (50), MYCN (47), MYCL1 (26), and MYCL2 (6) shows conservation of the leucine zipper and helix-loop-helix motifs and an upstream basic region within the gene family. These structures are thought to have a role in DNA binding and transcription regulation. Highly conserved residues including leucines in the zipper motif, hydrophobic residues in amphipathic helices, and hydrophilic residues in the region 5′ of helix 1 are defined by boxes.

difference in expression of MYCL2 may indicate developmental regulation of this gene. MYCL2 transcript was also detected in RNA from a lung adenocarcinoma and may play a role in development of this tumor type; however, the significance of a very low level of expression in this adenocarcinoma is not known.

Expression of MYC (8,14,27), MYCL1 (28), and MYCN (28) in testis tissue has previously been reported. In addition, MYCN RNA (28) and MYC protein (29) have been detected in human testicular seminomas by RNase protection and immunohistochemical analyses, respectively. Furthermore, expression of Myc in various cell types of normal murine testis has been characterized extensively (8,27). The amount of Myc RNA found in germ cells of adult mouse testis is much lower than that found in actively dividing somatic cells, including neonatal Sertoli and Leydig cells (8). In adult mouse testis, Myc transcripts are found mostly in mitotically active spermatogonia and in lower amounts in spermatocytes in the first stages of meiosis; Myc transcript is not seen in post-meiotic haploid germ cells (8,27). It has been suggested that the distinct pattern of expression of nuclear proto-oncogenes and transcription regulatory factors at the junction between mitosis and meiosis may indicate a regulatory role of these genes in transition from the proliferative state of germ cells to their differentiation into more mature cell types (8,14,27).

Transcripts of other proto-oncogenes, notably Mos, Abl, Int-1, Pim-1, and Nmyc, in addition to genes encoding structural and enzymatic products have been reported in the post-meiotic haploid germ cell population of the testis (15). Post-meiotically expressed genes such as testicular histone 2B, protamine, testis-specific α-tubulin, heat shock protein 70, and sperm-specific isozymes lactate dehydrogenase-X and phosphoglycerate kinase have been hypothesized to contribute to chromatin reorganization, and other morphological and functional changes taking place after meiosis, during post-meiotic spermiogenesis (15,30).

Distinct temporal and spatial patterns of expression of genes transcribed uniquely or in altered forms or amounts in the testis indicate an apparent regulation of these genes in specific cell types and stages of growth and/or differentiation. *In situ* localization of MYCL2 transcripts in the testis is needed to gain insight into their specific function in this tissue; these studies are currently in progress in our laboratory.

Antisense Transcription

Nucleotide sequences corresponding to opposite strand MYCL2 transcripts which we have analyzed do not contain any sizable open reading frame and are therefore not likely to be exons of genes encoded on the complementary strand of MYCL2, but could represent an intron of another gene. Alternatively, these antisense transcripts may serve regulatory functions. The phenomenon of antisense transcription has been studied extensively in prokaryotes and more recently in eukaryotic systems, elucidating its regulatory role in cellular functions such as RNA transcription, splicing, transport, stability, DNA replication, and translation; some of these functions are thought to be due to inhibitory effects of base pairing to complementary sense RNA (31−35). Other studies of antisense transcription have revealed the existence of different genes encoded on opposite strands of the same locus with various degrees of overlap in either their coding or non-coding regions (36−39). Bidirectional RNA polymerase II-directed transcription of opposite strands of the murine and hamster dihydrofolate reductase (Dhfr) gene from a GC-rich 5′ region lacking canonical TATAA and CAAT sequences has been reported (40,41).

Antisense transcription has been reported in various members of the myc family of proto-oncogenes. Nuclear runoff assays on human SCCL cell lines have shown antisense transcription of MYC, MYCN, and MYCL1 throughout different regions of each gene (42). In MYC, the most intense opposite strand transcription has been seen in the 5′ upstream region of the gene in HL60 (43), SCCL (42), and several murine cell lines including NIH3T3 cells (44). Significant antisense transcription of MYC has also been reported in exon 1, intron 1, intron 2, and exon 3, all of which are either spliced or include noncoding sequences in mature sense RNA (42,44). MYC polymerase II-directed antisense transcripts do not possess any long open reading frames, are thought to be unstable, and seem to be regulated independently of sense strand transcription (42−45). MYCN antisense RNA has been found in SCCL and neuroblastoma cell lines (46). The nonpolyadenylated fraction of this antisense RNA forms stable RNA:RNA duplexes *in vivo* selectively with only one of a number of different species of sense RNAs each possessing a different transcription start site, thereby increasing the half life of the sense RNA in the duplex (46). It has been speculated that the antisense transcripts in the myc family of proto-oncogenes may play a role in splicing of sense transcript, altering stability of sense RNA, or controlling availability of selected forms of sense RNA for translation (44,46).

MYCL2 Transcription Initiation

We have shown that MYCL2 may have multiple transcription start sites in a GC-rich region (~70% GC-rich in the 5′ flanking region), lacking appropriately spaced TATAA or CAAT boxes. A similar pattern of transcription has been demonstrated in MYCN (47−49) in contrast to other members of the myc gene family whose promoters are associated with classical TATAA and CAAT boxes (26,50). In addition, an SP1 recognition consensus site (51−54), GGGCGG, is located about 60-bp upstream of the most 5′ transcription start site in MYCL2; this distance is in the typical range of 40- to 70-bp upstream of transcription start sites. Numerous genes including MYC (3,50), MYCL1 (3,26), and herpes simplex virus thymidine kinase (55) have SP1 recognition sites in addition to typical TATAA or C-AAT motifs in their promoter regions. However, a number of eukaryotic genes, including MYCN (47−49), dihydrofolate reductase (Dhfr) (56,57), 3-hydroxy-3-methylglutaryl coenzyme A reductase (58), and hypoxanthine phosphoribosyltransferase (HPRT) (59) have GC elements in the absence of classical eukaryotic consensus motifs in their promoter regions. Deletion analyses have demonstrated that one of four GC boxes is sufficient to promote accurate transcription from the major *in vivo* transcription initiation site of the murine Dhfr gene (60). Interestingly, a number of genes, including MYCN (47−49), Dhfr (41,56,61), and HMGCoA reductase (58), which possess GC-rich promoters in the absence of canonical TATAA or C-AAT motifs show transcription initiation from multiple sites, a pattern resembling that seen in MYCL2.

Function of Intronless Genes

Although most known eukaryotic intronless genes are thought to be pseudogenes, there is increasing evidence for functional intronless genes, some of which possess characteristics of processed genes which have been reintegrated into the genome. A review of a number of these genes has revealed interesting parallels to MYCL2.

Similar to MYCL2, a number of functional intronless genes such as murine Zfa (62), phosphoglycerate kinase 2 (PGK2) (63,64), human pyruvate dehydrogenase 2 (PDHA2) (65), and chicken protamine (66) are expressed in significant amounts only in adult testis. N-myc 2, present in the woodchuck genome and thought to have arisen from reintegration of a spliced product of N-myc 1, has been implicated in pathogenesis of hepatocellular carcinoma (67). In addition, MYCN2 was shown to transform rat embryo fibroblasts in cooperation with activated HRAS(67), a phenomenon common to the myc family of proto-oncogenes (3).

The conservation of functional domains in intronless genes lends further support to their role in cellular processes. MYCN2 encodes a polypeptide which has retained the leucine zipper (68) and helix-loop-helix (69) motifs in its carboxy terminus (67). An analysis of the coding region of MYCL2 (6) reveals the conservation of these motifs in the expected positions in this gene (Fig. 8). Both of these structural motifs are thought to play a role in transcription regulation by involvement in protein dimerization and sequence specific DNA binding (69–73). The juxtapostion of the helix-loop-helix motif directly N-terminal to the leucine zipper motif is a unique arrangement characteristic to the myc gene family (74,75).

The leucine zipper motif found in MYCL2 displays only conservative amino acid substitutions from sequences of other myc gene family members and therefore retains capacity to code for a stable alpha helical structure with oppositely charged acidic and basic residues which could form ion pairs. Notably, one of five leucine residues in the chain of heptad repeats is substituted by a methionine residue in both MYCL1 and MYCL2. In addition, MYC and MYCN have substitution of one leucine to valine and alanine, respectively. It has been noted that conservative change in one leucine residue does not interfere with dimerization of Fos and Jun (76). Furthermore, among the amino acids which are structurally similar to leucine, methionine is thought to be the most compatible substitute for leucine in 'zipper' formation (70,76).

The helix-loop-helix structural motif of MYCL2 is consistent with two alpha helices containing amphipathic amino acid residues and highly conserved hydrophobic residues separated by a loop structure with a characteristic composition including proline, aspartic acid, asparine, and serine. Helix 1, helix 2, and the alpha helical leucine zipper motifs in MYCL2 do not show a single amino acid change to proline or glycine, residues which disrupt alpha helical structure. The helix-loop-helix motif is preceded by a basic region with highly conserved hydrophilic residues. These residues as well as those in the amphipathic helices (residues boxed in Fig. 8) are identical to, or highly conserved in MyoD, *Drosophila* daughterless, achaete-scute and twist genes (69). Interestingly, of the 32% dissimilarity in amino acid compostion between MYCL1 and MYCL2 in the helix-loop-helix motif and its 5′ basic region, there is only one amino acid change (arginine to lysine) within the evolutionarily conserved boxed residues.

ACKNOWLEDGEMENTS

We appreciate helpful discussions and review of the manuscript by Jack Sarid, Eric Sinn, Gary Tanigawa, and Ben Tycko. We thank George D. Sorenson and Olive S. Pettengill for the DMS 153 SCCL cell line and Bernard Collins for tumor tissues. This work was supported by grants from the National Institutes of Health CA 46737 (to C.C.M.) and R29-CA 49859 (to G.L.M.) and a Clinical Oncology Career Development Award (89-182) from the American Cancer Society (to G.L.M).

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[1]Where possible, gene symbols are in accordance with the guidelines for human gene nomenclature (1) and murine gene nomenclature (2); nomenclature for other species are in accordance with that in the published manuscript.