Chromosome localization and expression pattern of Lmyc and Bmyc in murine embryonal carcinoma cells

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Using Southern blot analysis of DNA from mouse-hamster somatic cell hybrids, we have mapped Lmyc and Bmyc, two members of the myc family of genes, to mouse chromosomes 4 and 2, respectively. Furthermore, we have compared the regulation of Lmyc and Bmyc expression under different growth conditions and during in vitro differentiation of the murine EC line F9 and considered the findings in relation to our previous studies on Nmyc and c-myc expression in the same line (Sejersen et al., 1987). Lmyc was down-regulated at an early stage of visceral endoderm differentiation, similarly to c-myc and Nmyc, while Bmyc was expressed at a constant low level at all stages. Lmyc, but not c-myc and Nmyc, was up-regulated in terminally differentiated visceral endoderm cells. Inhibition of protein synthesis by cycloheximide for 4 h induced a 70% increase in Lmyc and 30% increase in Bmyc transcript levels, indicating that the expression of these genes is negatively regulated by a short-lived protein. Mitogenic stimulation with insulin and transferrin did not affect Lmyc and Bmyc mRNA levels. Lmyc transcripts have a half life of 30 min, whereas the Bmyc transcript is highly stable, with a half life of 6 h. The half-lives of the c-myc and Nmyc transcripts have been estimated previously as 40 and 130 min, respectively.

Illegitimate activation of c-myc is believed to contribute to the genesis and/or progression of many different tumors. Juxtaposition of c-myc to an immunoglobulin locus and subsequent constitutive expression is regarded as an essential step in the genesis of mouse plasmacytoma, spontaneous immunocytoemia of the Louvain rat and human Burkitt lymphoma. Facsimile experiments with activated myc-constructs introduced into transgenic mice have confirmed the tumorigenic potential of constitutively activated c-myc and Nmyc in vivo (Adams et al., 1985; Rosenbaum et al., 1989). Amplification of c-myc may contribute to the progression of carcinomas (Little et al., 1983), sarcomas (Schwab et al., 1985) and leukemias (Asker et al., 1988; Dalla-Favera et al., 1982a; Sümege et al., 1985).

The isolation of Nmyc and Lmyc was based on their amplification in certain human tumors. Nmyc is amplified in neuroblastomas and in a fraction of small-cell lung carcinomas in a progression-related fashion (Brooks et al., 1986; Seeger et al., 1985). Lmyc is also amplified in a subset of small cell lung carcinomas (Brooks et al., 1986; Neu et al., 1985). Thus, either c-myc, Nmyc or Lmyc is amplified in small cell lung carcinomas with a variant, more invasive and more highly metastatic phenotype (Brooks et al., 1986). This suggests a potential functional equivalence of the three genes, in relation to their progression-promoting potential in this tumor. Rmyc and Pmyc have been isolated on the basis of the homology to the third Lmyc exon (De Pinho et al., 1987a) and Bmyc on the basis of its homology to the second c-myc exon (Ingvarsson et al., 1988). C-myc, Nmyc, Lmyc and Rmyc are equally efficient in the rat embryo fibroblast co-transformation assay (Birrer et al., 1988; De Pinho et al., 1987a; De Pinho et al., 1987b; Yancopoulos et al., 1985).

In contrast to the homologies between the coding regions, there is no homology between the large non-coding first exons of c-myc, Nmyc and Lmyc, and the non-coding regions of their third exon (De Pinho et al., 1986; Legouy et al., 1987). These regions are highly conserved between the corresponding human and mouse genes, however, suggesting that they may play an important regulatory role, perhaps in relation to the tissue specific expression pattern (De Pinho et al., 1986; De Pinho et al., 1987b; Legouy et al., 1987). The first exon of c-myc has been proposed to participate in post-transcriptional regulation of message stability (Eick et al., 1985; Rabbits et al., 1985). Moreover, the demonstration of a transcriptional block in c-myc and Lmyc, located within the first exon of c-myc, suggests a rapidly acting control mechanism for the expression of the gene (Krystal et al., 1988; Eick et al., 1986).

In view of the documented participation of myc genes in tumor associated chromosomal changes, it is of inter-

Introduction

The myc family consists of six functional genes; c-myc, Nmyc, Lmyc, Rmyc, Pmyc, and Bmyc (De Pinho et al., 1987a; Ingvarsson et al., 1988). C-myc encodes a nuclear protein that is regularly expressed in a wide variety of proliferating cells. Nmyc and Lmyc expression is limited to certain developmental stages and cell types (Zimmerman et al., 1986). Many tissues express Bmyc, independently of their developmental stage (Ingvarsson et al., 1988). During the embryonic development of the mouse and rat, c-myc, Nmyc, Lmyc, and Bmyc are expressed in different regions, suggesting independent regulation (Ingvarsson et al., 1988; Zimmerman et al., 1986). C-myc, Nmyc and Lmyc have a similar structure and encode similarly sized nuclear proteins (Legouy et al., 1987; Evan et al., 1988; Henriksson et al., 1988). Both c-myc and Nmyc proteins bind to single- and double-stranded DNA in vitro (Ramsay et al., 1986) and both genes can stimulate DNA replication in certain systems (Classon et al., 1987; Cavaliere et al., 1988). The c-myc protein may act as a transcriptional activator (Lech et al., 1988; Iguchi-Ariga et al., 1988; Dean et al., 1987).
est to extend their mapping to additional species. In this study we have determined the chromosomal localizations of the mouse Lmyc and Bmyc genes. Their position differs from the known location of other mmyc family members. We have also examined their expression in murine embryonal carcinoma (EC) cells under different growth conditions and during induction of differentiation. Growth conditions were varied by a serum starvation and growth factor induction. Differentiation was induced by retinoic acid, a potent inducer of visceral endoderm formation, which was shown to be concomitant with the down-regulation of c-myc and Nmyc (Sejersen et al., 1987). Our results show that c-myc, Nmyc, Lmyc, and Bmyc are differentially regulated in embryonal carcinoma cells.

### Results

#### Chromosome localization

To localize Lmyc and Bmyc in the mouse, we screened HindIII or PstI digested mouse-Chinese hamster hybrid panels that segregate mouse chromosomes, with a rat Lmyc probe (second exon) (Ingvarsson et al., 1987; Figure 1) and pRM44, a rat Bmyc probe (Ingvarsson et al., 1988). The results indicate that the 2.5 kb and 1.6 kb PstI mouse Lmyc-specific bands were present in hybrids EASS-7c, EBS9 GTG, I-3-2D-1d aza and I-18A-2a aza and absent in 9 other hybrid lines: EASS-17c, EASS-5c, EASS-2a, EASS-4a, EBS2, EBS11 HAT, EBS10 and RTM10 HAT (which is a mouse/rat cell hybrid). The 8.5 kb BamHI mouse Bmyc-specific band was present in hybrids EASS-5c, EASS-2a, EASS-7c, EBS2, EBS9 GTG, I-3-2D-1d aza, EBS10 and EASS-17c (that contains only mouse chromosome 2) and absent in two other hybrid lines: EASS-4a and EBS11 HAT. Figure 2 gives examples of positive and negative hybrids. Comparison with the chromosomal segregation data (Table 1 and 2) showed that mouse chromosomes 2 and 4 gave a consistent pattern. We have therefore concluded that the mouse Bmyc and Lmyc loci are on chromosomes 2 and 4, respectively.

#### Expression of Bmyc in rat brain sections

We have previously shown that Bmyc expression is highest in fetal and newborn brain compared to other rat tissues. Adult brain provides the greatest contrast, since c-myc is turned off and Bmyc is expressed at a high

### Table 1

<table>
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#### Discordant hybrids

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The numbers of hybrids that are concordant (+/+ or −/−) and discordant (+/− or −/+), with the mouse Bmyc sequence are given for each chromosome. Data on rearranged chromosomes or chromosomes present in fewer than 10% of cells were excluded.

### Table 2

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<td>−/−</td>
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</tr>
<tr>
<td>+/−</td>
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<tr>
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#### Discordant hybrids

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<td>11 12 12 12 11 12 11 12 12 10 11 12 12 11 12 12 11 12</td>
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The numbers of hybrids that are concordant (+/+ or −/−) and discordant (+/− or −/+), with the mouse Lmyc sequence are given for each chromosome. Data on rearranged chromosomes or chromosomes present in fewer than 10% of cells were excluded.
level (Ingvarsson et al., 1988). To explore this difference in more detail we isolated RNA from several adult rat brain sections. A single transcript of 1.3 kb was detected in total RNA and/or the polyA+ fraction from all rat brain sections examined (Table 3, Figure 3) as well as in the mouse embryonal carcinoma cell line F9 (Figure 4). Figure 3 shows a blot of total RNA from adult rat brain sections hybridized to a 0.5 kb PstI fragment of Bmmyc (Ingvarsson et al., 1988). The transcript was detected in hippocampus, hypothalamus, striatum, cortex, cerebellum, midbrain and medulla. Expression was highest in the cerebellum. The relative expression in the other tissues is shown in Table 3. The c-myc expression was negative in all of the adult brain sections compared to the fetal expression, except for a slight expression in hypothalamus, striatum and midbrain (Figure 3, Table 3).

Expression of Lmmyc and Bmmyc in F9 cells

In order to investigate the relationship between expression of the genes Lmmyc and Bmmyc and cell proliferation, we analyzed their expression in serum-deprived and insulin/transferrin stimulated F9 EC cells. Exponentially growing F9 cells express both 3.9 kb Lmmyc and 1.3 kb Bmmyc (Figure 4), as well as the 2.9 kb Nmmyc and 2.4 kb c-myc mRNA (Sejersten et al., 1987; Jakobovits et al., 1985). The effect of growth inhibition on Lmmyc and Bmmyc expression in F9 cells was studied by exposing subconfluent cultures of F9 cells to 0.5% FCS for 48 h. The decrease in DNA synthesis and the amount of [3H]TdR incorporated into DNA during a 1 h pulse was decreased by about 90% following serum deprivation (Sejersten et al., 1987). The relative abundance of Lmmyc mRNA, measured by densitometer scanning of radiographs, was decreased by 90% after serum deprivation.

Table 3 Relative Bmmyc and c-myc expression as percentage of the highest expression

<table>
<thead>
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<th>Bmmyc</th>
<th>c-myc</th>
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<tr>
<td>Hippocampus</td>
<td>66% (2)</td>
<td>2% (2)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>39% (3)</td>
<td>9% (3)</td>
</tr>
<tr>
<td>Striatum</td>
<td>75% (3)</td>
<td>9% (3)</td>
</tr>
<tr>
<td>Cortex</td>
<td>38% (3)</td>
<td>9% (3)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>100% (3)</td>
<td>4% (3)</td>
</tr>
<tr>
<td>Midbrain</td>
<td>44% (3)</td>
<td>7% (3)</td>
</tr>
<tr>
<td>Medulla</td>
<td>45% (2)</td>
<td>1% (2)</td>
</tr>
<tr>
<td>Brain, 3d old</td>
<td>99% (3)</td>
<td>100% (3)</td>
</tr>
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The brain sections were taken from adult rat. For each independent experiment, the intensity of a particular myc mRNA band in a given section was calculated as a percentage of the signal intensity generated with RNA from the most strongly expressing tissue/section (cerebellum in the case of Bmmyc and total brain from newborn rat in the case of c-myc), which was arbitrarily set at 100%. Each value is an average of 1 to 3 independent experiments (given in parentheses).

Figure 2 (a) Southern blot analysis of Chinese hamster DNA (CH), mouse DNA (M), and four hybrid cell clones cleaved with PstI and hybridized to the pl2.5 probe. The mouse specific germ line bands are at 2.5 and 1.6 kb. The Chinese hamster specific band is at 1.8 kb. (b) Southern blot analysis of Chinese hamster DNA (CH), mouse DNA (M), and four hybrid cell clones cleaved with HindIII and hybridized to the pRM44 probe. The mouse specific germ line band is at 8.5 kb and the Chinese hamster specific band are at 5.8 kb and 2.9 kb.

Figure 3 Expression of Bmmyc and c-myc in several adult rat brain sections and fetal brain, representative Northern blot analysis. The filters were sequentially hybridized to three different probes; pMm-c-myc 54, GAPDH (for standardizing the RNA amount on the filters), and pRM05. Hy, hypothalamus; S, striatum; Co, cortex; Ce, cerebellum, Mi, midbrain; Hi, hippocampus; Me, medulla; F, fetal brain; A, adult brain.
viation while Bmyc mRNA was unaltered (Figure 4). GAPDH expression was not affected by the serum starvation (Figure 4). Differentiation-specific α-fetoprotein (AFP) mRNA was not synthesized after serum deprivation (Sejersen et al., 1987).

In order to analyze further the relationship between growth induction and regulation of Lmyc and Bmyc expression we added serum-free medium with insulin (1 μg ml⁻¹) and transferrin (5 μg ml⁻¹) to serum-deprived F9 cells. Insulin and transferrin induce DNA synthesis in F9 EC cells (Rizzino & Crowley, 1980; Sejersen et al., 1987). The Lmyc; Bmyc, and Nmyc do not show rise in expression in contrast to the strong rise in c-myc expression induced by 4 h of insulin/transferrin (Figure 4, Sejersen et al., 1987).

It has been previously reported that both c-myc and Nmyc are down-regulated in F9 cells differentiating into visceral and parietal endoderm by a post-transcriptional mechanism (Sejersen et al., 1987; Jakobovits et al., 1985; Campisi et al., 1984). Northern blot analysis shows that the down regulation of Lmyc, c-myc and Nmyc associated with visceral endoderm differentiation followed different kinetics, while the Bmyc mRNA was unaffected (Sejersen et al., 1987; Figure 4). The c-myc and Nmyc show some variations in the amount of mRNA at day 4 and/or 10 of RA induced differentiation in different experiments. These variations may reflect slight difference in the time when cells are refed with serum containing medium. An upregulation of Lmyc at day 10 of RA induced differentiation is consistent in several experiments.

**Stability of Lmyc and Bmyc mRNA in F9 cells**

The c-myc and Nmyc mRNAs have a rapid turnover in F9 cells with a half-life of about 40 and 130 min, respectively (Sejersen et al., 1987). We have determined the intracellular turnover of Lmyc and Bmyc mRNA in proliferating F9 cells by following the changes in the steady-state levels of both transcripts at various time points after the addition of actinomycin D, a potent inhibitor of RNA transcription. Figure 4 shows that the Lmyc mRNA disappeared much more rapidly than the Bmyc transcript in actinomycin D-treated F9 cells. A quantitative analysis of the turnover kinetics of steady-state levels of Lmyc and Bmyc in F9 cells revealed half-lives of approximately 30 min for Lmyc and over 6 h for Bmyc. GAPDH transcripts served as a control and were not affected by 6 h of actinomycin D treatment.

In view of the above findings, we wanted to test if the expression of Lmyc and Bmyc were regulated by a labile protein. We examined the effect of protein synthesis inhibition by cycloheximide on the steady-state levels of Lmyc and Bmyc mRNA before and after RA induction. Figure 4 shows that cycloheximide treatment for 3 h
enhanced the abundance of \( \text{Lmyc} \) and \( \text{Bmyc} \) mRNA approximately 70% and 30%, respectively, in both undifferentiated and RA-induced F9 cells. In a similar experiment the \( c\text{-m} \) mRNA shows twofold enhancement, while the levels of \( N\text{myc} \) did not undergo an appreciable change during this time period (Sejersen et al., 1987).

**Discussion**

**Mapping of \( \text{Lmyc} \) and \( \text{Bmyc} \) to mouse chromosomes 4 and 2**

We have mapped the mouse \( \text{Lmyc} \) and \( \text{Bmyc} \) loci to chromosomes 4 and 2, respectively. It has previously been shown that \( c\text{-m} \) is on chromosome 15 (Dallafavera et al., 1982b) and \( \text{Nmyc} \) is on chromosome 12 (F. Alt, personal communication). The localization of the four genes of the \( \text{myc} \) family on different chromosomes confirms that they represent separate loci, as already shown in rats and humans. These chromosomal assignments are in agreement with the detection of conserved banding homology between rat and mouse chromosomes (Nesbitt, 1974; Yoshida, 1978). The \( \text{myc} \)-carrying mouse chromosomes 2, 4 and 12 show a homologous banding pattern to the rat chromosomes 3, 5 and 6, and they also share corresponding gene loci (Ingvarsson et al., 1987). The short arm of human chromosome 1 (1p) and mouse chromosome 4 share \( \text{Lmyc} \) and seven other loci (Neu et al., 1985; Lalley et al., 1987). Seven of these genes map to rat chromosome 5, which shows a certain banding homology with mouse chromosome 4. Five genes on mouse chromosome 2 have their counterparts on human chromosome 9 (9a) and two of them also map to rat chromosome 3 (Lalley et al., 1987). Deletion of mouse chromosome 2 is particularly frequent in myeloid leukemias (Hayata et al., 1983). It would be of interest to know whether this is associated with any changes in the \( \text{Bmyc} \) gene.

**Bmyc expression**

The detection of \( \text{Bmyc} \) in the rat, mouse, human and hamster genome (Ingvarsson et al., 1988; Figure 2) and the conservation of its open reading frame in comparison with \( c\text{-m} \) (Ingvarsson et al., 1988) suggest that it is a functional gene. This is confirmed by the expression of \( \text{Bmyc} \) in many tissues. In this respect, \( \text{Bmyc} \) is similar to \( c\text{-m} \), but there are also some important differences. \( \text{Bmyc} \) is expressed at uniformly high levels in all fetal tissues and during subsequent postnatal development in contrast to the stage specific expression of \( c\text{-m} \) (Ingvarsson et al., 1988; Figure 3). The rat brain expresses high levels of \( \text{Bmyc} \), with less than 3-fold variations between different regions while the \( c\text{-m} \) expression is only detectable at a low level (Figure 3).

**Lmyc and Bmyc expression in F9 cells**

\( \text{Lmyc} \) and \( \text{Bmyc} \) expression in F9 cells is differentially regulated in response to serum deprivation, growth factors, and/or induction of visceral endoderm differentiation. \( \text{Lmyc} \), and also the previously investigated \( c\text{-m} \) and \( \text{Nmyc} \) were down-regulated in serum starved cultures, whereas \( \text{Bmyc} \) remained fully expressed.

Insulin induced a marked elevation in \( c\text{-m} \) expression, as expected on the basis of its previously known mitogenic action in F9 cells (Rizzino & Crowley, 1980). The \( \text{Nmyc} \), \( \text{Lmyc} \) and \( \text{Bmyc} \) do not respond to this mitogenic induction.

In RA induced cultures, \( c\text{-m} \), \( \text{Nmyc} \) and \( \text{Lmyc} \) expression decreased prior to the inhibition of DNA synthesis and the appearance of visceral endoderm differentiation whereas the expression of \( \text{Bmyc} \) did not change. This is in line with our previous finding concerning the lack of tissue or stage specificity of \( \text{Bmyc} \) expression in developing rat tissues (Ingvarsson et al., 1988; Figure 3). The upregulation of \( \text{Lmyc} \) at day 10 of RA induced differentiation may reflect negative cross-regulation between the \( \text{myc} \) family members (Cleveland et al., 1987).

The experiments on transcript stability and response to cycloheximide revealed further differences concerning the regulation of the \( \text{myc} \) genes in F9 cells. \( c\text{-m} \), \( \text{Lmyc} \) and \( \text{Bmyc} \) showed an increased expression after cycloheximide treatment for 3 h, indicating that their expression may be negatively regulated by a short-lived protein. \( \text{Nmyc} \) was not influenced. \( c\text{-m} \) and \( \text{Lmyc} \) have short lived transcripts, \( \text{Bmyc} \) an extraordinarily long half-life, while that of \( \text{Nmyc} \) is intermediate.

**Materials and methods**

**Hybridization probes**

The \( \text{myc} \) probes were as follows: (a) \( p\text{M} \), the 1.9 kb internal HindIII fragment from the cDNA mouse \( c\text{-m} \) containing the second exon and parts of the first and the third exons (Stanton et al., 1983), (b) \( p\text{D} \), a 0.5 kb internal XhoI-BamHI fragment from the cDNA human \( \text{Nmyc} \) containing the second exon (Kohli et al., 1986), (c) \( \text{Bmyc} \), human 0.8 kb SmaI-PvuII fragment containing the 3' part of the second exon and 1.0 kb PvuI-EcoRI fragment containing the second intron (Neu et al., 1985); mouse \( \text{Lmyc} \) exon 2, a 0.5 kb Nael fragment; \( p\text{Rf} \), a 1.2 kb BamHI-PvuII fragment containing the coding part of the third exon and a 1.5 kb XbaI-EcoRI fragment containing the noncoding part of the third exon (Legouy et al., 1987); \( p\text{L} \), a 2.5 kb EcoRI-BamHI fragment from the genomic rat \( \text{Lmyc} \) including the second exon and second intron (Ingvarsson et al., 1987; Figure 1) and \( p\text{L} \), a 1.4 kb HindIII fragment from the genomic rat \( \text{Lmyc} \) including the third exon (Figure 1), (d) \( \text{pR} \), a 0.5 kb PstI fragment containing the part of the rat \( \text{Bmyc} \) gene that shows \( c\text{-m} \) first intron and second exon homology (Ingvarsson et al., 1988) and \( p\text{R} \), a 1.3 kb PstI fragment containing the part of the rat \( \text{Bmyc} \) gene that shows \( c\text{-m} \) second and third exon homology (Ingvarsson et al., manuscript in preparation) and GAPDH, a 550 bp XbaI-HindIII fragment containing the human glyceraldehyde-3-phosphate dehydrogenase gene (Tso et al., 1985). The probes were [\( ^{32} \text{P} \)]-labeled by the ‘oligonucleotide’ method (Feinberg & Vogelstein, 1983) to a specific activity of \( 7 \times 10^{8} \text{cpm \mu g}^{-1} \).

**Cell hybrids and Southern blotting**

The hybrid series I, EAS and EBS were derived from fusion between mouse and Chinese hamster cells (Francke et al., 1977; Francke & Taggart, 1979). Ten micrograms of hybrid and parental control cell DNA were digested with HindIII or PstI. DNA fragments were separated by electrophoresis in 0.8% agarose and transferred to Hybond-N (Amersham) nylon filter. Hybridization with oligolabelled probes, washing and autoradiography were carried out as described (Barton et al., 1986).
Cell cultures
F9 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), nonessential amino acids, nucleosides, and sodium pyruvate. For induction of differentiation to visceral endoderm they were treated with 5 x 10^{-8} M RA for 10 days in cellassociated dishes. F9 cells were serum deprived by 48 hour culture in DMEM with 0.5% FCS. For mitogenetic stimulation insulin (Sigma) and transferrin (Sigma) were added to serum deprived cultures at 1 μg·ml^{-1} and 5 μg·ml^{-1}, respectively. Transcription was inhibited by actinomycin D (5 μg·ml^{-1}, Sigma) in serum-supplemented DMEM. Protein synthesis was inhibited by addition of cycloheximide (Sigma) at 10 μg·ml^{-1} to the culture medium.

Isolation of RNA and RNA blotting
RNA was isolated by hot phenol extraction (Edmonds & Caramella, 1969) or by the guanidine isothiocyanate/lithium chloride/phenol method described by Cathala et al., (1983). Total or poly(A)+ RNA (Aviv & Leder, 1972) was denatured, fractionated by formaldehyde-agarose (11%) gels and transferred to Hybond-N nylon membranes (Amersham). Filters were prehybridized at 42°C overnight in a solution containing 6 x SSC, 1 x Denhardt's, 1% SDS, and 100 μg·ml^{-1} boiled herring sperm DNA. Hybridization reactions were incubated at 42°C for 18 hour in a solution consisting of 6 x SSC, 1% SDS, 50% formamide, 5% dextran sulfate and 100 μg·ml^{-1} herring sperm DNA. [32P]-labeled probe was added at a concentration of 2 x 10^6 cpm·ml^{-1}. The membranes were washed twice at room temperature in 2 x SSC, 0.1% SDS for 30 minutes, for one hour at 65°C in 0.2 x SSC, 0.1% SDS and for 5 minutes at room temperature in 0.1% SSC. The filters were exposed to Fuji X-ray film with intensifying screens for 2-5 days. The radioactively labeled probe was removed by washing the filters 3 times for 10 minutes each in a boiling solution of 0.2 x SSC and 0.1% SDS. Filters were prehybridized immediately after stripping for 4 hours and stored at 4°C in prehybridization solution. Densitometry was performed on a LKB Ultrascan XL laser densitometer using Gaussian fit analysis.

References

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