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# Translocation affects normal c-*myc* promoter usage and activates fifteen cryptic c-*myc* transcription starts in plasmacytoma M603

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## ABSTRACT

Plasmacytoma M603 contains one normal, nontranslocated c-myc gene and one translocated c-myc gene in which c-myc exon 1 is juxtaposed with the immunoglobulin heavy chain enhancer and c-myc exons 2 and 3 are juxtaposed with Cα. We find that steady-state c-myc RNA levels are 2-4 fold elevated in M603 relative to normal liver or spleen and that these transcripts originate predominantly if not exclusively from the translocated c-myc gene. Although both promoters on the nontranslocated c-myc gene are repressed, the proximal promoter, P1, is active on the translocated 5' c-myc region which is juxtaposed with the immunoglobulin heavy chain enhancer. The 3' portion of the translocated c-myc gene is transcribed from fifteen cryptic start sites and spliced at aberrant donor and acceptor splice sites, thereby generating a mixture of transcripts with different, abnormal 5' untranslated regions. Although the reason that translocation activates the cryptic c-myc starts in M603 is not completely understood, we show that truncation of the c-myc gene is not sufficient to activate cryptic transcription sites.

## INTRODUCTION

Alteration in the cellular homologue of the avian myelocytomatosis virus transforming gene, c-myc, appears to be one of several events necessary for malignant transformation in many tumors (1-3). As distinct from the ras family of oncogenes (4,5), changes in c-myc which are important in oncogenesis involve alterations of gene expression rather than alterations of protein coding sequences (2). The expression of c-myc in normal cells is highly regulated--it correlates with the growth state of the cells (6), varies during the cell cycle (7), and is differentially expressed in different tissues (1). The c-myc gene is transcribed from two promoters, P1 and P2, located about 150 bp apart (8,9, this paper) and appears to be repressed by a labile repressor protein (6). Since these data suggest that normal c-myc regulation is quite complex, it is not surprising that there are many points where this finely tuned regulation can be perturbed. It has been found that in different tumors the c-myc gene is altered in various ways: i) retrovirus insertion brings transcriptional promoter or enhancer elements

near c-myc in avian bursal lymphomas (10,11) and murine lymphomas (12,13), ii) the c-myc gene is amplified in several tumors (14-17), and iii) chromosomal translocations occur within or near c-myc in human Burkitt lymphomas (18-22), murine plasmacytomas (23-26) and rat plasmacytomas (27). How these alterations ultimately affect c-myc expression and how changes in c-myc expression affect cell growth control are poorly understood at present.

Our attention has focused on c-myc gene expression in tumors where one c-myc gene has undergone chromosomal translocation. Translocation in Burkitt lymphomas and murine plasmacytomas juxtaposes the c-myc gene with immunoglobulin genes; however, the sites of translocation relative to c-myc and to specific immunoglobulin gene regions vary. In many Burkitt lymphomas, the translocation juncture does not interrupt the c-myc gene or its immediate flanking sequences. In tumors of this type there is evidence that translocation causes mutations in DNA sequences distant from the translocation juncture. Many of these changes occur in the 5' flanking region of c-myc, prompting the suggestion that changes in the 5' flanking region may alter a c-myc repressor binding site (6, 28). However, in murine plasmacytomas c-myc is most frequently "decapitated" so that all or part of the 5' flanking region and the first untranslated exon are removed from the two coding exons (29). In these cases, c-myc transcription cannot be initiated at the normal sites and presumably cannot be normally repressed. Thus, different c-myc regulatory mechanisms appear to operate depending on the site of translocation relative to the c-myc gene.

In this paper we report a detailed analysis of c-myc transcription in plasmacytoma M603 where translocation "decapitates" one c-myc gene (26). Our results show that the normal, nontranslocated c-myc gene in M603 is expressed very little if at all and that translocation alters transcription of both 5' and 3' segments of the translocated gene.

## METHODS

DNA blots. Ten to fifteen micrograms of total genomic DNA was digested to completion with restriction enzymes, size-separated on 1% (w/v) agarose gels, and transferred to nitrocellulose filters (30). Filters were prehybridized, hybridized and washed using the conditions of Wahl et al. (31). Washed filters were exposed to preflashed Kodak XAR-5 film at -70°C with a Dupont Cronex Lighting Plus intensifying screen for 1-3 days.

RNA Isolation. Nuclear RNA for Northern blot analysis and cytoplasmic RNA for S1 nuclease analysis was isolated as previously described (32). 18-81

cells from suspension culture were washed with cold phosphate buffered saline and lysed with 0.1% NP40. M603 solid tumors were excised and immediately frozen and pulverized in liquid nitrogen and then lysed in a buffer containing 0.1% NP40. Nuclei were pelleted and detergent washed (33,34) before being broken in a Dounce homogenizer in lysis buffer. Cytoplasmic and nuclear fractions were phenol extracted and ethanol precipitated. Nuclear RNA was treated for 15 min at 37°C with 10 µg/ml RNase-free DNase, extracted and ethanol precipitated. All buffers up to this stage in the preparations contained 10 mM vanadyl ribonucleoside complex, an inhibitor of RNases (35). Total RNA was isolated by mixing washed cultured cells or tumor tissue ground in liquid nitrogen with guanidine thiocyanate solution and banding in CsCl (36). Poly(A)$^+$ RNA was selected in all cases by passage over an oligo dT cellulose column.

RNA Blots. Nuclear or total poly(A)$^+$ RNA, 10-50 µg per lane, was separated on formaldehyde agarose gels as described (32). Ribosomal RNA was run in outside lanes to provide size standards. Blotting and hybridization conditions were those described by Thomas (37).

S1 Nuclease Analysis. S1 nuclease analysis was carried out according to the method of Berk and Sharp (38) and Weaver and Weissmann (39). 5' end-labeled fragments were prepared using bacterial alkaline phosphatase and polynucleotide kinase as described by Maxam and Gilbert (40). 3' end-labeled fragments were prepared by end-filling using the Klenow fragment of DNA polymerase I, also as described by Maxam and Gilbert (40).

Hybridization reactions were carried out in a total volume of 10 µl, containing 10-50 ng of DNA and 20-25 µg of poly(A)+ cytoplasmic RNA in 80% (v/v) recrystallized deionized formamide, 0.4 N NaCl, 0.04 M Pipes, pH 6.4 and 1 mM EDTA. Control reactions contained 30 µg of yeast tRNA. The reaction mixtures were heated to 90°C for 10 min and then incubated at 56°C for 4 hr. Hybridizations were stopped by the addition of 200 µl ice cold S1 buffer containing 200-600 U/ml S1 nuclease as described (32). S1 reactions were incubated at 20°C for 2 hr prior to precipitation of the protected DNA fragments and electrophoresis on 8% polyacrylamide sequencing gels containing 7 M urea. Size standards on these gels were Hpa II cleaved pBR322 DNA which had been labeled by end-filling with $\alpha^{32}$P dCTP using the Klenow fragment of DNA polymerase I.

Primer Extension. Primer extension reactions were carried out using a 117 bp 5' end labeled Taq I fragment as primer by a modification of published procedures (41,42). RNA-primer hybrids were formed by mixing 240 ng primer

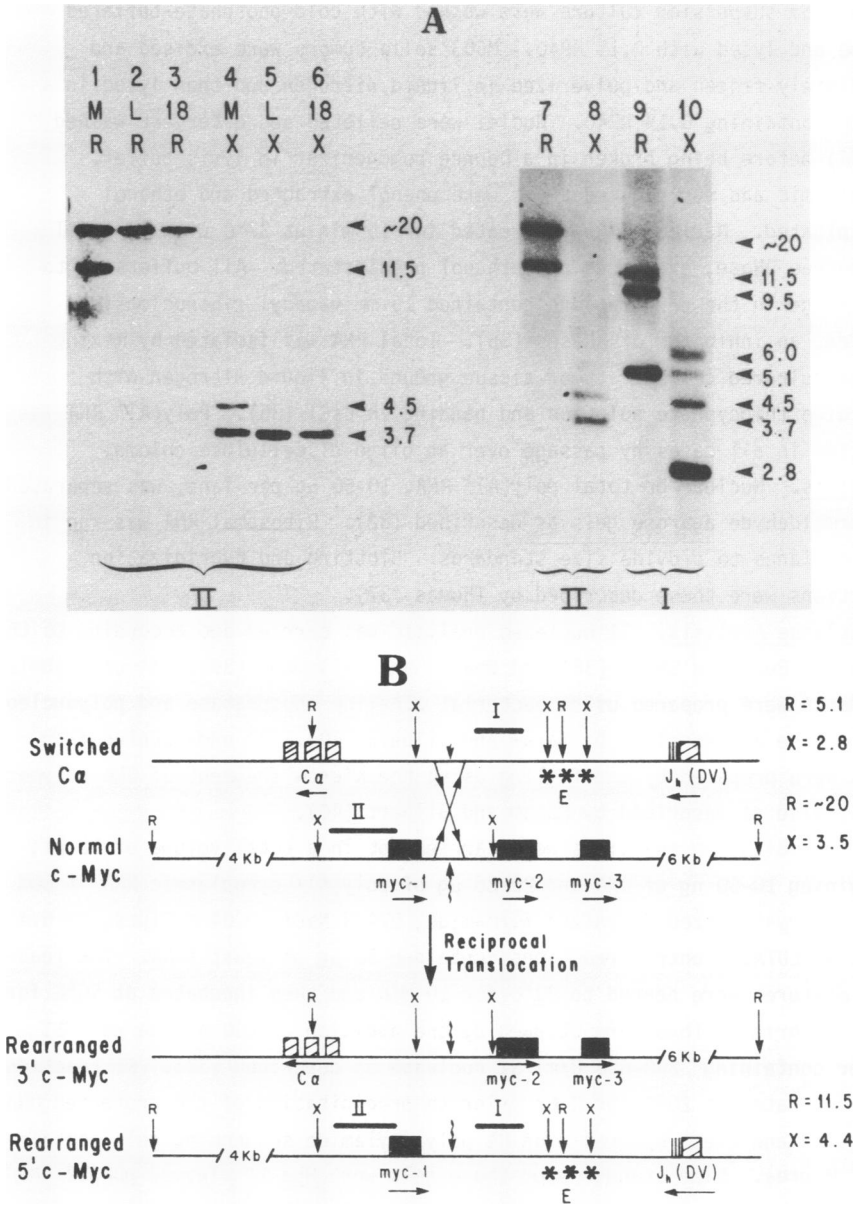


Fig. 1. Southern blot analysis of c-myc translocation in M603.
A. M603 (M), Balb/c liver (L) and 18-81 (18) DNAs were restricted with EcoR1 (R) or Xba I(X) as indicated. Lanes 1, 4, 7-10 are M603 DNA, lanes 2 & 5 are liver and lanes 3 & 6 are 18-81. Probes I and II indicated on map in B. Band sizes indicated in kilobases.

B. Map of reciprocal c-myc/alpha translocation event. Rearranged 3' c-myc has been cloned from a genomic library of M603. Rearranged 5' c-myc configuration was deduced from genomic Southern blots in A. The possible presence of D and V gene segments has not been confirmed but is suggested if the translocation involved an active alpha gene. EcoR1 = R, XbaI = X. Immunoglobulin enhancer region indicated by asterisks and E. Arrows below exons show direction of transcription. Wavy arrows show translocation break points. I & II indicate probes used on Southern blots in A.

and 30 µg poly(A)$^+$ M603 RNA in 20 µl total hybridization mixture which was 80% deionized and recrystallized formamide, 0.4 M NaCl, 0.04 M Pipes, pH 6.4, 1 mM EDTA. The mixture was placed in a sealed capillary and heated to 85°C for 15 min then incubated at 55°C for 16 hr. The hybrids were ethanol precipitated, resuspended in synthesis buffer and incubated at 42°C for 60 min. Synthesis buffer contains 50 mM Tris (pH 8.3), 140 mM KCl, 8 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 50 µM EDTA, 1 mM each deoxynucleotide triphosphate, 50 U/µl RNasin and 20 U/µl avian myeloblastosis reverse transcriptase (Promega).

L-Cell Transfection. A vector containing the region of the 3' rearranged c-myc gene shown in Fig. 7a was co-transfected with a vector containing the herpes virus thymidine kinase gene into thymidine kinase deficient mouse L cells (tk$^-$L cells) using the calcium phosphate method (43). Three HAT resistant lines were isolated.

## RESULTS

### Translocation juxtaposes the 5' flanking region and first exon of c-myc with sequences upstream of the mu switch site.

We have previously characterized the structure of the c-myc gene which is translocated to Cα in M603 by analysis of genomic clones (25,26). This gene contains most of the first intron and the two coding exons but lacks the 5' non-coding exon. Before analyzing c-myc transcription, we wished to confirm a previous report (29) that M603 belonged to the class of plasmacytomas in which reciprocal translocation juxtaposes the 5' region of c-myc with sequences upstream of the mu switch site (Sµ). Fig. 1a shows the results of genomic Southern blots of normal liver and tumor DNAs which were hybridized with a subcloned probe specific for the 5' portion of c-myc (II in Fig. 1b). With Eco R1 and Xba I digests, the 5' c-myc probe hybridizes to one band, 20 and 3.5 kb respectively, in liver DNA (lanes 2 and 5) and Abelson virus transformed pre-B cell 18-81 DNA (lanes 3 and 6), demonstrating that the c-myc gene is not translocated in these cell types. However, this probe hybridizes to one normal and one translocated band in the M603

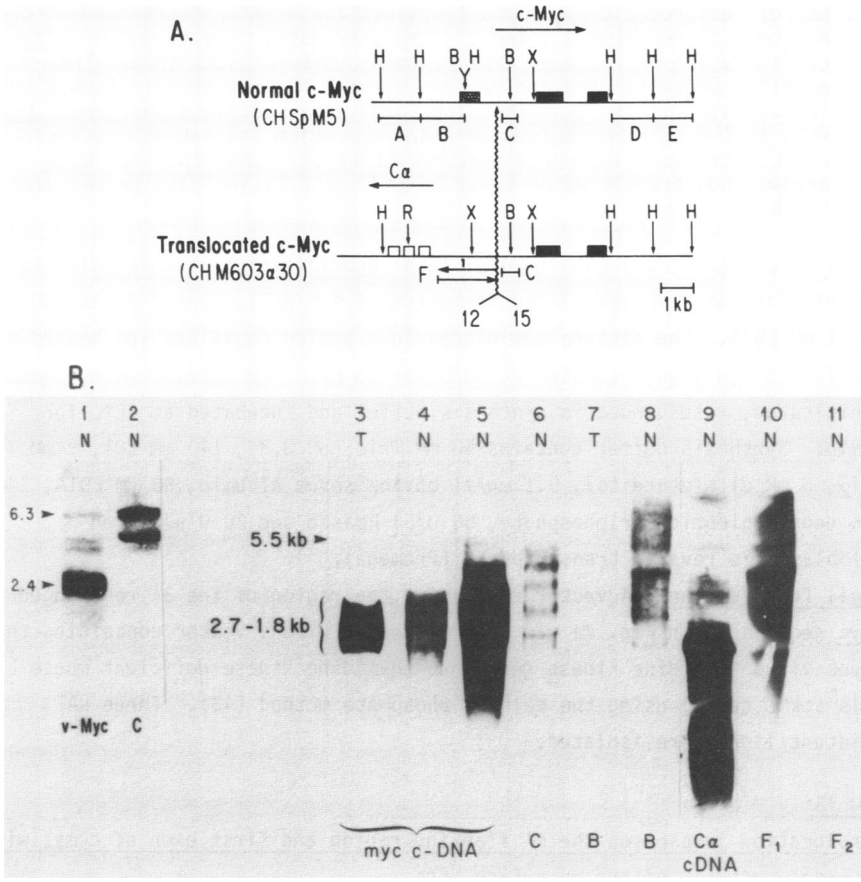


Fig. 2. Northern analysis of c-myc transcripts in M603

A. Map of normal and translocated c-myc genes showing subcloned fragments used as probes for Northerns (A-F). F1 and F2 are single stranded specific probes cloned in M13. H = Hind III, B = Bam HI, X = Xba I.

B. Northern analysis of 18-81 poly(A)$^+$ nuclear (N) RNA (lanes 1-2) and M603 poly(A)$^+$ nuclear (N) and total (T) RNA (lanes 3-11). Probes used are indicated on map in A and are written under each lane. The v-myc probe used in lane 1 contains avian virus myc sequences (60). Sizes in kilobases. Lanes 1,3,4 and 6 were exposed 10-16 hrs; lanes 2,5,7,8 and 11 were exposed 2-3 days; lanes 9 & 10 were exposed 4 hrs.

DNA (lanes 1 and 4), demonstrating that the 5' portion of c-myc which remains on the shortened chromosome 15 is present in a rearranged context.

The c-myc translocation could have occurred with a germline Cα gene, juxtaposing the first c-myc exon with sequences 5' of Sα or with a Cα gene which had undergone class switching, juxtaposing the first exon with sequen-

ces 5' of Sμ. To distinguish between these two possibilities, Southern analyses were performed using two restriction enzymes and three probes. Xba I and Eco R1 digests of M603 DNA were hybridized with the 5' c-myc probe (II in Fig. 1b) and with probes specific for sequences 5' to either Sμ or Sα; we expected that one of these probes would hybridize to the same fragments as the 5' c-myc probe. The 5' Sα probe did not show any strongly hybridizing bands and the faintly hybridizing bands did not correspond to bands which hybridized with the 5' c-myc probe (data not shown). However, the 5' Sμ probe (I in Fig. 1b) hybridized to a 4.5 kb Xba I fragment and to an 11.5 kb Eco R1 fragment (lanes 10 and 9). These sizes agree with the predicted sizes for a reciprocal translocation with a Cα gene which had previously undergone class switching and also correspond with the Xba I and Eco R1 fragments which hybridize with the 5' c-myc probe (lanes 8 and 7). Thus, we conclude that in M603, a reciprocal translocation juxtaposed the first c-myc exon with the immunoglobulin heavy chain enhancer (44-46) and Jh region, as depicted in Fig. 1B.

Northern analyses of c-myc transcription in M603

When the steady-state levels of c-myc hybridizing transcripts in M603 and mouse spleen and liver were compared by RNA dot blot analysis, we found that total c-myc transcripts in M603 were approximately 2 fold higher than liver and 4 fold higher than spleen (data not shown). We used Northern analyses to determine whether these transcripts arose from the normal c-myc gene, the translocated c-myc gene, or both. We also determined if the c-myc promoters on the translocated 5' fragment were active. In order to compare c-myc transcripts in M603 with c-myc transcripts in a tissue where the gene is not translocated, 18-81 RNA was analyzed in parallel experiments. A map of normal and translocated c-myc genes showing the probes used for these studies is presented in Fig. 2A.

As shown in Fig. 2B, c-myc transcripts in nuclear RNA from 18-81 contain a 6.3 kb precursor, processing intermediates and mature message of about 2.4 kb (lane 1). Probe "C" from the intervening sequence between the first and second c-myc exons hybridizes with precursors but not with mature message (lane 2), as expected for an intervening sequence probe.

In contrast, when M603 RNA was hybridized with a c-myc cDNA probe containing sequences from exons 2 and 3, we found transcripts in both nuclear and total RNA which were very heterogenous, ranging in size from approximately 2.7-1.8 kb (lanes 3 and 4). A 5.5 kb precursor was seen faintly when lane 4 was exposed longer (lane 5) and also when probe "C" was used (lane

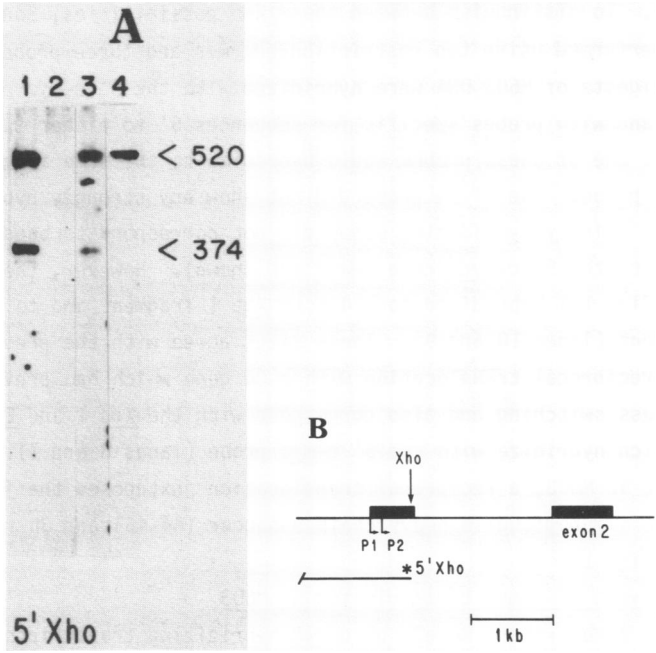


Fig. 3. S1 nuclease analysis of transcripts from normal myc promoters P1 and P2 in M603.
A. Lane 1, 18-81 cytoplasmic RNA; lane 2; yeast tRNA, lane 3, Balb/c liver cytoplasmic RNA; lane 4, M603 cytoplasmic RNA. Sizes (in nucleotides ± 2) indicated on right. 5' Xho probe shown on map in B.
B. Map of c-myc showing 5' end labeled Xho fragment used in A.

6). Failure to detect a 6.3 kb precursor suggested that transcripts from the untranslocated c-myc gene were decreased relative to translocated transcripts. To search specifically for nontranslocated transcripts, total M603 RNA, which is predominantly cytoplasmic in origin, was hybridized with the 5' c-myc probe, probe "B", which would detect transcripts from P1. No hybridization was observed with this probe (lane 7). (This is an appropriate probe to use because S1 nuclease experiments shown in Fig. 3 show that P2 transcripts are absent in M603.) Thus, we estimate that transcripts from the nontranslocated c-myc gene, undetectable on this blot, are at least 10-20 fold lower than translocated c-myc transcripts. Although probe "B" did not hybridize to total RNA, long exposure of nuclear RNA revealed hybridization of heterogenous sizes (lane 8), showing that transcripts containing this sequence exist in the nucleus but are not detectable in the cytoplasm. Since they are heterogenous in size and are not correctly spliced and trans-

ported, we conclude that these transcripts originate from the translocated exon 1 which is juxtaposed with the heavy chain enchancer and Jh regions (Fig. 1b).

To delimit the 5' boundary for initiation of translocated c-myc transcripts, we cloned a Pst I fragment extending 1.4 kb 5' from the translocation juncture into M13 in both orientations to obtain strand-specific probes (Fig. 2a). Probe F1, specific for the Cα strand, hybridized to alpha precursor transcripts and processing intermediates but not to the mature alpha mRNA because it is an intervening sequence probe. In contrast, probe F2, specific for the c-myc strand, showed no detectable hybridization to M603 nuclear RNA (lane 11). Lack of hybridization with this probe indicates that the translocated c-myc transcripts do not initiate within or 5' of probe F2. This is consistent with the shorter 5.5 kb precursor seen in lanes 5 and 8. However, since probe "C" hybridizes to nuclear precursors and to a small subset of mature c-myc transcripts (lane 8), we conclude that at least part of the translocated transcripts initiate within or 5' to the "C" probe.

In summary, the Northern analyses of M603 c-myc transcripts show that: i) there is little or no transcription from the normal gene, ii) there is low transcription from the translocated c-myc promoters but the transcripts are not present in cytoplasm, and iii) transcripts from the translocated c-myc gene are heterogeneous, initiate 3' to the translocation juncture, and a subset of them contain sequences represented by "C" probe. In order to characterize these transcripts in more detail, S1 nuclease and primer extension experiments were performed.

P1 is active and P2 is repressed in M603

The Northern data presented above show there is a low level of transcription from the normal 5' c-myc promoter and suggest that it is from the translocated gene segment, not from the normal c-myc gene. We used S1 analyses with a 5' end-labeled Xho I probe to analyze the usage of both c-myc promoters more precisely (Fig. 3). The labeled Xho I site is located within the first c-myc exon (3b) and transcripts initiating from P2 protect a 374 bp fragment, while transcripts initiating from P1 protect a 520 bp fragment (Fig. 3a). The results show that in normal liver and in 18-81 cells transcripts from the two promoters are present in approximately equal amounts (Fig. 3a, lanes 1 and 3). However, in M603, P2 is completely repressed; only transcripts from P1 were detected (lane 4).

Translocated c-myc transcripts in M603 initiate at fifteen cryptic sites 5' to the second c-myc exon.

S1 nuclease analysis was used to study the translocated c-myc trans-

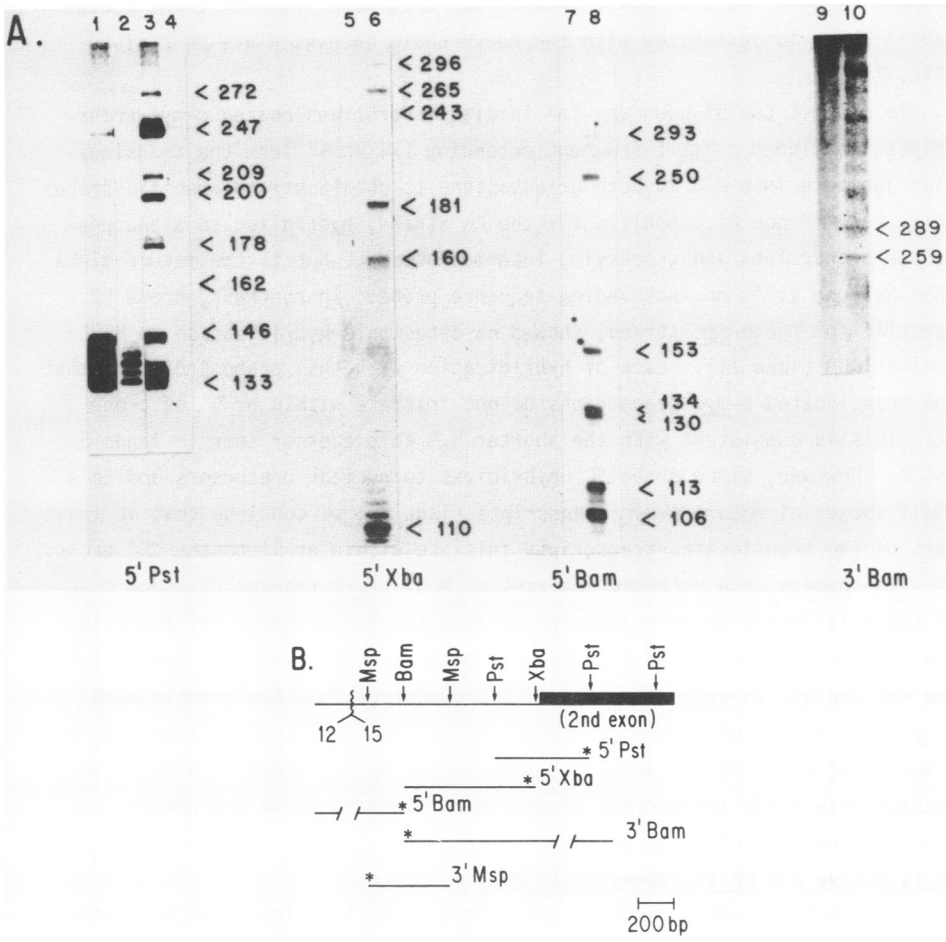


Fig. 4. S1 nuclease mapping of cryptic transcription start sites and aberrant splice sites in the first c-myc intron.

A. Probes used indicated under groups of lanes. The 5' Pst probe was 402 bp; the 5' Xba probe was 799 bp; the 5' Bam probe was 2.65 kb and the 3' Bam probe was 3.25 kb. Lane 1 and 2, 18-81 cytoplasmic RNA; lanes 3,6,8,10, M603 cytoplasmic RNA; lanes 4,5,7,9, yeast tRNA. 25 µg of RNA was used for each lane. Lanes 2-10 exposed 16 hrs; lane 1 is the same as lane 2 only exposed 3 days. Sizes of bands (in nucleotides ± 5) indicated for each group.

B. Map of translocated c-myc gene showing $^{32}P$ end labeled fragments used in A.

cripts in M603. Three 5' end-labeled probes covering the region from the second c-myc exon to the translocation juncture and two 3' end-labeled probes were used for these studies (Fig. 4b). Several distinct protected

fragments were obtained using each 5' end-labeled probe (lanes 3,6,8); in sum, 21 different fragments, corresponding to 20 different 5' sites were protected with M603 RNA. The 133 bp fragment protected with the Pst I probe corresponds to splicing at the second c-myc exon acceptor splice site. This fragment, as well as the family of fragments ranging from 135-146 bp which could result from incomplete S1 digestion or splicing upstream of the regular site, are protected with 18-81 RNA (lanes 1 and 2) as well as with M603 RNA. No 3' fragments were protected using the 3' Msp I probe (data not shown). Two distinct protected fragments were obtained using the 3' Bam probe (lane 10) and we interpret these sites to be cryptic donor splice sites. Seven 5' sites upstream of the aberrant donor splice sites were detected using the 5' Bam probe (lane 8). We interpret these sites to be cryptic transcription start sites. Initiation in this region, and splicing of sequences from the region into mature transcripts was predicted by the hybridization of probe "C" with the M603 5.5 kb precursor and with mature mRNA (Fig. 2, lane 6). However, we were unable to determine from the S1 data alone whether the remaining eleven 5' sites downstream of the cryptic donor splice sites which were mapped with the Pst I probe (lane 3) and the Xba I probe (lane 6) were cryptic acceptor splice sites or transcription initiation sites. It is interesting to note that only one of these (Pst I 247 bp fragment) was protected by 18-81 RNA (lane 1).

In order to determine whether these eleven 5' ends were aberrant acceptor splice sites or transcription initiation sites, primer extension reactions were carried out using a 117 bp Taq I fragment from the second c-myc exon as primer (Fig. 5b). Aberrant primer extension products can result when reverse transcriptase pauses or stops before the end of the mRNA. However, we reasoned that coincidence of 5' ends mapped by primer extension and S1 protection would be strong evidence for transcription initiation at that site, whereas, absence of a primer extension product corresponding to an S1 site would suggest an aberrant acceptor splice site. Since primer extension products larger than 490 bp are ambiguous because they could result either from unspliced transcripts or from spliced transcripts initiated 5' of the Bam site, only primer extension products smaller than 490 bp were considered in this analysis. Seven discrete extension products, as well as larger products which were not accurately sized, were obtained using M603 RNA (Fig. 5a). The positions of six primer extension products map +/- 5 bp of the corresponding 5' site mapped by S1 nuclease (Fig. 5b); this degree of variation reflects the limits of resolution on our gels.

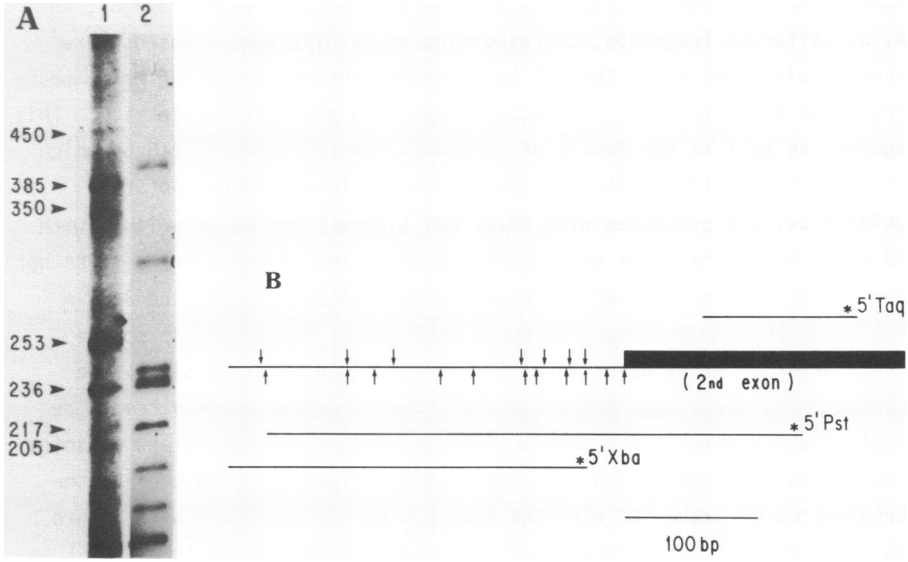


Fig. 5. Primer extension mapping of cryptic start sites in region 5' of second myc exon.
A. Lane 1, sequencing gel lane with products of primer extension reaction; lane 2, pBR322 cleaved with HpaII and end filled as size markers.
B. Map of primer extension and S1 fragment sizes. Arrows below line mark 5' ends of fragments (within 5 nucleotides, centered about arrow) protected by 5' Pst and 5' Xba probes in S1 experiments of Fig. 4A. Arrows above line mark 5' ends of fragments generated by primer extension using the indicated 5' end labeled Taq I fragment as primer.

Correspondence between S1 nuclease 5' sites and primer extension products is good evidence that these sites represent transcription initiation sites. The 350 bp primer extension product which did not correspond with a S1 site may represent a strong stop due to secondary structure in the mRNA (we note DNA sequence that this region has the potential to form a short hairpin structure). Five 5' sites detected with S1 nuclease did not have a corresponding primer extension product; we conclude they are acceptor splice sites. These include the normal acceptor splice site and the family of sites just upstream of it as well as three cryptic sites further upstream. One of these upstream sites corresponds to the 247 bp Pst I protected fragment from S1 analysis which was also protected with 18-81 RNA. We conclude that it is an aberrant acceptor splice site which can be used at low levels in the absence of c-myc translocation. Due to difficulties sizing larger primer extension products, we were unable to determine if the two largest

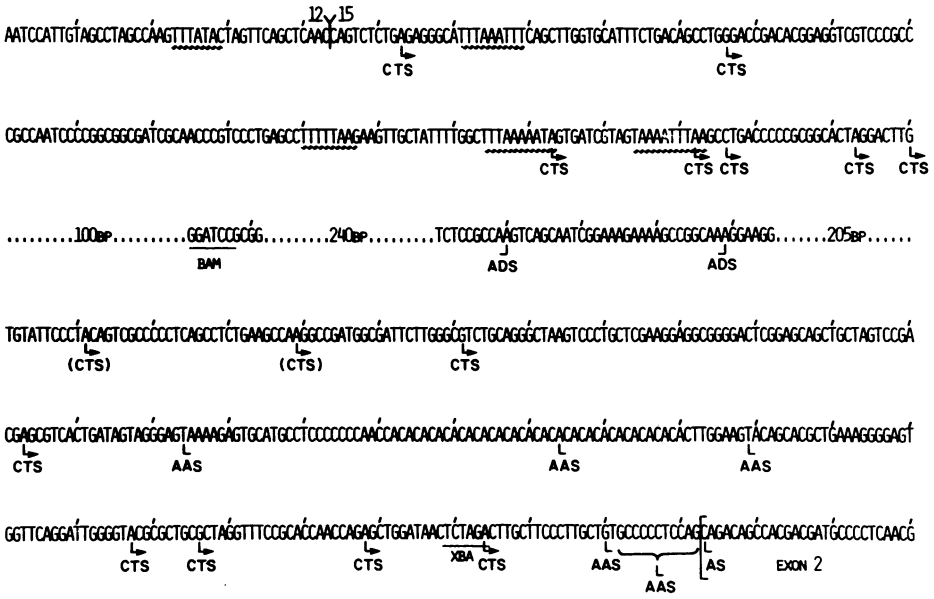


Fig. 6. DNA sequence of translocated c-myc from region of 12/15 chromosome translocation juncture to exon 2. Cryptic transcription start (CTS) sites, aberrant donor splice (ADS) sites, and aberrant acceptor splice (AAS) sites mapped by S1 nuclease protection and primer extension experiments in M603 (Fig. 3B) are shown. Size of protected fragments can be determined to within about five nucleotides (centered on the indicated nucleotide) using our standard size markers.

Xba I protected fragments were start or splice sites but we believe that they are likely to be start sites because faint larger primer extension products of approximately the correct sizes were observed.

The identification of seven cryptic transcription starts 5' of the Bam HI site which are spliced from aberrant donor splice sites is consistent with hybridization of some c-myc transcripts with probe "C". However, the eight additional cryptic transcription start sites closer to c-myc exon 2 which were confirmed with the primer extension studies would not hybridize with probe "C". This is consistent with the Northern results (Fig. 2b, lane 6) showing that not all translocated c-myc mRNAs hybridized with probe "C".

Fig. 6 shows the DNA sequence of this region, as determined by N. Takahashi, R. Barth and L. Hood (in preparation), with cryptic transcription start sites (CTS), aberrant acceptor splice sites (AAS), and aberrant donor splice sites (ADS) indicated. It is interesting that the region near the translocation juncture is AT rich and in fact 5' of seven cryptic start

sites in this region, there is a TATA region, typical of eurkaryotic promoters (47). However, this is not true for the eight cryptic start sites nearer to the second c-myc exon; none of them have TATA sequences upstream. Only 2/4 aberrant acceptor splice sites have the canonical AG of acceptor splice sites and 1/2 aberrant donor splice sites has the canonical GT sequence (47). Overall, based on consensus sequences for normal transcription initiation and splice sites, it appears that at least half of the aberrant sites used for translocated c-myc transcripts are distinctly atypical. In other plasmacytomas, 5' ends which were interpreted to be transcription initiation sites have been mapped by S1 analysis (52,55) but these do not correspond to the ones which we find in M603, suggesting that different tumors may transcribe and process translocated c-lyc genes differently.

A complex mixture of c-myc transcripts containing abnormal 5' ends is predicted from the 15 cryptic initiation sites, two aberrant donor splice sites, 4 aberrant acceptor splice sites and one normal acceptor splice site which we have mapped. However, analysis of the sequence in this region shows that there are translational termination codons in all three reading frames throughout the intervening sequence region where the 5' ends originate. Therefore, the translocated c-myc transcripts have abnormal 5' untranslated regions, but they do not encode altered myc protein.

Removal of the first c-myc exon and flanking sequence is insufficient to activate the cryptic promoters and splice sites.

There is evidence that the normal c-myc gene is repressed and that the repressor binding site may be located within or 5' to the first exon (6, 48). However, the exact nature and location of this repressor and its binding site is not understood. Our results showed that transcripts from the cryptic promoters found in M603 were not present in 18-81 RNA where there is no translocated c-myc gene. Thus, we asked if the 5' region might act in cis to repress transcription from the cryptic promoters, allowing their activation upon removal of the region.

We constructed a vector which contained the M603 translocated c-myc gene (exons 2 and 3) but extended only to the Xba I site located 510 bp 5' to the translocation juncture (Fig. 7b). This vector was co-transfected with the herpes virus thymidine kinase gene into thymidine kinase deficient (tk-) mouse L cells and three tk+ lines were isolated for study. Southern analysis showed that each of the lines contained approximately 5-20 copies of the truncated c-myc gene (data not shown). RNA from each line was isolated and analyzed by S1 nuclease analysis. Fig. 7a compares the patterns

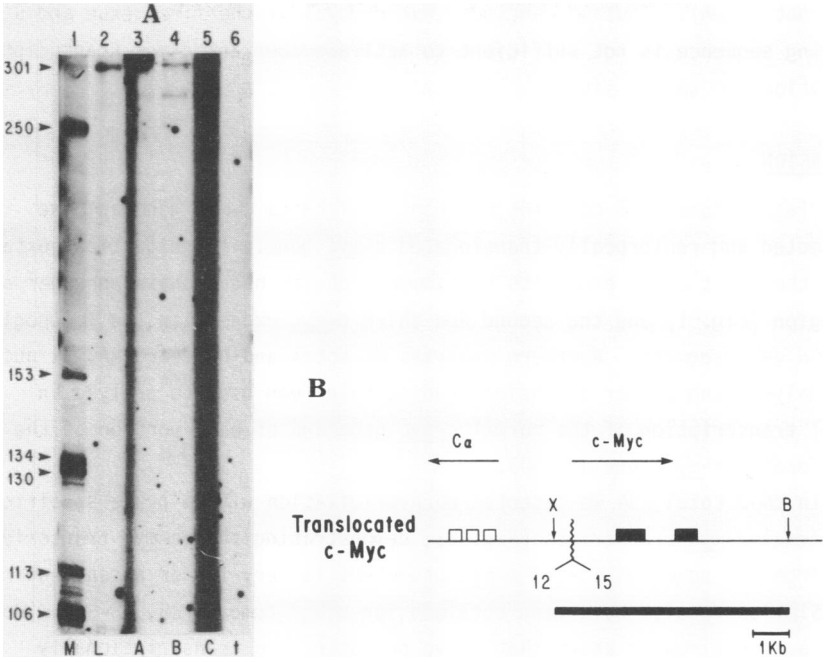


Fig. 7. Analysis of L-cells transfected with truncated c-myc gene.
A. Map showing region (solid bar) of M603 α30 subclone transfected into tk-L cells to obtain lines A,B,C (see B) containing the truncated myc gene.
B. S1 nuclease analysis of transfected L-cell lines using 5' Pst fragment of Fig. 4B as probe. Lane 1, M603(M) total RNA; lane 2, tk-L cell total RNA; lanes 3-5, lines A,B and C respectively; lane 6, tRNA control. Band sizes in nucleotides show fragments of M603 previously identified as cryptic transcription start sites, Fig. 4a, lane 8.

obtained with RNA from M603, tk-L cells, and three L-cell transfected lines using the 5' Bam probe to detect initiation sites near the translocation juncture. A 301 bp fragment was protected in untransfected L cells (lane-2) and in all three transfected lines. This is the predicted size that a precursor transcript from the normal c-myc gene would protect (see Figs. 2 and 4) and indicates that the endogenous c-myc gene is transcribed normally in the transfected lines. However, the transfected lines show very little transcription from cryptic c-myc sites 5' to exon 2 even though the copy number of the transfected truncated gene was several times higher than the endogenous gene. Similarly negative results were obtained using the 5' Pst I probe to detect cryptic transcription start sites near the second exon

(data not shown). Thus we conclude that removal of the first exon and 5' flanking sequence is not sufficient to activate aberrant c-myc transcription initiation and splice sites.

## DISCUSSION

Plasmacytoma M603 contains one nontranslocated c-myc gene and one transected and reciprocally translocated c-myc gene. Translocation juxtaposes the first c-myc exon with the immunoglobulin heavy chain enhancer and Jh region (Fig. 1) and the second and third c-myc exons with the immunoglobulin Cα gene segment. Northern analyses of total and nuclear RNA, S1 nuclease analyses and primer extension studies have been used to analyze in detail transcription of the normal c-myc gene and of each portion of the translocated c-myc gene in M603.

In M603 total RNA we detected no hybridization with a probe specific for normal c-myc transcripts (Fig. 2), demonstrating that c-myc transcription from the nontranslocated c-myc promoters is very low or absent entirely. Similar results have been obtained for other tumors (28, 48-51). However, a recent report shows that c-myc promoters are transcriptionally active in normal proliferating B cells (52), suggesting the lack of transcription from the normal c-myc genes in Burkitt lymphomas and plasmacytomas does not reflect normal c-myc regulation, but reflects c-myc repression which is unique to these tumors. It may be that repression of the normal c-myc gene in tumors containing a translocated c-myc gene is caused by feedback inhibition resulting from aberrant expression of c-myc from the translocated gene. It has been suggested (6,48,53) that high levels of myc protein could cause, either directly or indirectly, repression of c-myc transcription. This suggestion is consistent with the fact that the nontranslocated c-myc gene is not repressed in the Raji line which produces a mutant myc protein from the translocated allele (54).

Regardless of the mechanism responsible for repressing transcription from the normal c-myc gene, it is noteworthy that one of the two translocated c-myc promoters is not repressed in M603 (Figs. 2 and 3). Both promoters are used about equally in normal liver and in cell line 18-81 where there is no c-myc translocation (Fig. 3) and both appear to be repressed on the nontranslocated gene in M603. If a repressor protein is involved in this regulation, as suggested (6,48,53), one would expect this repressor to act in trans and to repress transcription from both translocated c-myc promoters as well as both nontranslocated c-myc promoters. Our results show

that P1 and P2 are differentially regulated and stggest that some cis-acting effect of translocation 3' to the first exon is able to overcome transcriptional repression for P1 but not for P2. In cases where translocation occurs 5' to the first c-myc exon preferential usage of P1 relative to P2 has also been observed (28). It may be that the same mechanism activates P1 regardless of whether translocation is 5' or 3' to the promoters. In M603 the immunoglobulin heavy chain transcriptional enhancer element (44-46) is brought within 3 kb of the c-myc promoters and may be involved in P1 activation.

Transcripts containing c-myc exon 2 and 3 are elevated in M603 relative to normal mouse liver or spleen and originate predominantly or exclusively from the 3' portion of the c-myc gene translocated to the Cα region. A striking finding in our studies is that transcripts of this "decapitated" c-myc gene initiate from at least 15 discrete cryptic start sites; 5' leaders are spliced from two cryptic donor splice sites to three or four cryptic acceptor splice sites as well as to the normal acceptor splice site. Previous studies on other "decapitated" c-myc genes have used S1 nuclease analysis to map sites 5' of the second exon which were assumed to be cryptic promoters (28,52,55,56). In this study we demonstrated using primer extension reactions in conjunction with S1 nuclease studies that most of the sites we mapped by S1 analysis were indeed cryptic transcription start sites, but a few were cryptic acceptor splice sites (Figs. 4 and 5). In addition to being more numerous than those found in other tumors, the cryptic starts in M603 map to different locations. Based on these facts and the observation that at least half of the cryptic sites do not contain sequences typical of normal eukaryotic transcription initiation sites (Fig. 6), we conclude that translocation of c-myc to Cα causes a strong but non-specific activation of discrete transcription sites in the first c-myc intron and in different tumors different sites are activated.

What is the mechanism responsible for this unusual transcriptional activation in the first c-myc intron? The cryptic promoters are not active in tissues lacking translocated c-myc genes and no sequence changes were found in the intervening sequence region when the translocated gene from M603 was compared to a normal c-myc gene cloned from mouse sperm DNA (Takahashi, N., Barth, R and Hood, L., in preparation). Therefore activation appears to result from alterations of some cis-acting control which is capable of acting at a distance. One simple possibility is that removal of the first c-myc exon and its 5' flanking region is sufficient to activate

the cryptic promoters in the first intron. This could occur if transcription initiating at P1 and P2 and proceeding through the first intron was normally sufficient to repress the cryptic sites by the mechanism of promoter occlusion (57). Alternatively, the 5' region might contain some negative regulatory element capable of working at a distance either directly or by abolishing the effect of downstream enhancer elements (58). However, by transfecting a truncated c-myc gene into mouse L cells and demonstrating there was little transcription from cryptic sites in the first intron, we have ruled out models based solely on negative regulation from the first exon and 5' flanking sequences. Therefore, it is necessary to consider the action of some positive regulatory mechanism in activating these cryptic sites. This could be a cis-acting element in the translocated Cα region or a trans-acting factor from a lymphoid cell environment or both.

In another study we have recently shown that there is no transcriptional enhancer element located within an 11 kb region surrounding the cryptic c-myc promoters in the translocated c-myc gene in M603 (E. Kakkis, M. Mercola, J. Prehn and K. Calame, submitted). This eliminates the possibility that another enhancer element within the region tested could be reponsible for activating the cryptic promoters. Croce and co-workers have used somatic cell hybrids to demonstrate that activation of translocated c-myc genes from Burkitt lymphomas requires a lymphoid cell environment (49,59). It seems likely that chromosome 12 sequences combined with a lymphoid cell environment may be required to activate transcription from the cryptic c-myc sites. The mechanism of this intriguing cis-acting effect is unknown but is currently being investigated in our laboratory.

How does transcription of "decapitated" c-myc contribute to the transformed state of plasmacytoma cells? The transcripts contain abnormal 5' untranslated ends but do not encode altered myc protein. It may be, as suggested by Stanton et al. (55), that the abnormal 5' untranslated sequences lead to higher levels of myc protein in the cell. This could result from more efficient translation or increased cytoplasmic stability of translocated c-myc mRNAs; although recent experiments (60,61) suggest that a change in translational efficiency is less likely. However, we performed a computer search for stable local secondary structures in normal c-myc mRNA and in several putative translocated c-myc mRNAs. The results show a very stable (-105 kcal) hairpin structure involving the translation initiation codon which could form in the normal c-myc mRNA. Similar hairpins involving the initiation codon are less favored (a typical valud was -65 kcal) in putative

translocated mRNAs. Thus, loss of the potential to form such a stable secondary structure could alter the cellular fate of abnormal RNAs and contribute to the overall deregulation of myc. Alternatively, it is reasonable to assume that the cryptic promoters are constituitively activated after translocation and that expression from them does not vary during the cell cycle. It may be that failure to regulate c-myc expression during the cell cycle is sufficient to contribute to the malignant state or that lack of cell cycle regulation in conjunction with increased protein levels from aberrant transcripts is causal in oncogenesis.

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