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Strong transcriptional activation of translocated c-myc genes occurs without a strong nearby enhancer or promoter

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ABSTRACT

We have studied the transcriptional activation of translocated c-myc genes in murine plasmacytomas in which the translocation juncture occurs within the first intron of c-myc and juxtaposes c-myc with the immunoglobulin C_α gene segment. It has been widely suggested that a novel transcriptional enhancer element located near the C_α gene segment might activate the translocated c-myc gene. We have carried out an extensive search for such an element and find no significant transcriptional enhancer activity in a 22 kb region encompasing the translocation junction, C_α gene segment and regions 3' of C_α. We also find that the cryptic promoter region of the translocated c-myc gene is a very weak promoter of transcription. Despite this evidence against the presence of strong transcriptional regulatory elements, the translocated c-myc gene locus is transcribed at high rates that are 25->100% of that measured for the highly active immunoglobulin genes in murine plasmacytomas. These data suggest the presence of a novel type of strong activator of transcription in the murine heavy chain locus.

INTRODUCTION

The alteration of cellular proto-oncogenes by chromosomal translocation is one type of genetic defect commonly associated with malignancies (1). In human Burkitt lymphomas and murine plasmacytomas, the c-myc proto-oncogene is consistently involved in reciprocal chromosomal translocations with the immunoglobulin (Ig) loci (1,2). The translocation results in deregulated expression of the translocated c-myc gene in these tumors. The specific effects of this deregulation on cellular function are not understood, although some recent work supports a role for the c-myc protein in DNA replication (3,4). Recent studies show that transcriptionally activated c-myc genes will predispose transgenic mice to the formation of tumors (5,6). Therefore, the alteration in c-myc expression following chromosomal translocation probably constitutes an important step in the genesis of Burkitt lymphomas (7) and murine plasmacytomas. Understanding the mechanisms responsible for the deregulated expression of translocated c-myc genes is thus an important goal.

Studies of the translocated c-myc gene in plasmacytomas show that the c-

myc gene is usually broken in either the first exon or first intron and reciprocally translocated with the immunoglobulin heavy chain locus at heavy chain switch sites (8-12). The translocation usually results in the juxtaposition of the coding exons of c-myc with heavy chain constant region gene segments in a 5' to 5' orientation. An example of this structure is found in the plasmacytoma M603, an IgA producer with a c-myc translocation to the alpha constant region (ref. 10; Fig. 1). The translocation of the c-myc gene results in deregulated levels of truncated c-myc transcripts which are initiated at a heterogeneous set of cryptic sites primarily within the first intron (12-15). Transcripts from the normal c-myc allele are undetectable in these tumors (12-15). Although post-transcriptional mechanisms certainly contribute to deregulation (16), we have decided to focus on B cell-specific mechanisms which operate to activate transcription of translocated c-myc genes from the cryptic start sites.

There are a number of important characteristics of the activation which should be considered in formulating any model for this process. The transcriptional activation of translocated c-myc genes occurs at multiple cryptic start sites on both strands of the DNA (12,14,15,17). Somatic cell hybrid studies demonstrate that the expression of translocated c-myc genes occurs only in mature B cells (18-20). The somatic cell hybrid experiments also demonstrate that truncation of c-myc genes is not sufficient in itself to activate transcription since truncated c-myc genes are not expressed in fibroblasts or lymphoblastoid cells (18-20). Similarly, truncated c-myc genes introduced into cells by transfection are not expressed unless a transcriptional enhancer element is included in cis (15,21,22). Finally, the translocated c-myc gene has an open, DNase I-sensitive chromatin conformation that is the same as that observed for the highly expressed Ig genes (23). In contrast, the normal c-myc gene is relatively DNase I-insensitive, like an inactive gene locus (23).

Cis-acting sequences within the Ig locus are generally presumed to mediate the B cell-specific transcriptional activation of translocated c-myc genes. Transcriptional enhancer elements are a type of cis-acting regulators that might be responsible for this activation since they are known to activate transcription of heterologous promoters independent of orientation and distance (24). Enhancers often display tissue specificity which would be consistent with the observed B cell-specific activation of translocated c-myc genes (25,26). However, in most murine plasmacytomas, the Ig sequences adjacent to translocated c-myc sequences consist of the switch region, alpha constant

region gene segments and sequences 3' to the alpha constant region. With few exceptions (22,27,28), the known heavy chain enhancer is not adjacent to c-myc and no other transcriptional activating sequence is known in this region.

To explore the possibility that a novel transcriptional enhancer exists near the translocated c-myc gene, we have performed an extensive search for transcriptional enhancers, using both transient and stable transfections, of a 22 kb region of the murine alpha constant region/c-myc translocation juncture. The results, showing no detectable enhancer activity, strongly suggest that no enhancer exists in this region and that activation of the translocated c-myc gene occurs by another mechanism. We also found that the cryptic start region alone was a very weak transcriptional promoter as measured by transient transfection. Surprisingly, measurements of transcription rates revealed that translocated c-myc genes are highly transcribed, at rates comparable to that of Ig genes. Thus the strong transcriptional activation of translocated c-myc genes in plasmacytomas appears to occur in the absence of strong promoters or detectable enhancers.

MATERIALS AND METHODS

Cell and tumors lines Tumors MOPC 603, TEPC 1017, MOPC 173, M104E, and HOPC1 were obtained from Litton Bionetics (National Cancer Institute contract NO1-CB-25584) and passed subcutaneously in CxD2 F1 mice. The cell lines P3X63-Ag8 and S107 were maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Irvine Scientific) and 20 micrograms per ml gentamycin.

Phage and plasmid cloning The phage library was a kind gift of Drs. G. Siu and L. Hood. The filters were screened using the 3' C alpha probe (probe B, Fig. 1). Two phage, Ch3'C_{α}8B and Ch3'C_{α}12A contained the most sequence 3' of C alpha and were studied further. We discovered that Ch3'C_{α}8B was not representative of the genome and had undergone a recombination during the original cloning process. Ch3'C_{α}12A was unstable during large scale preparation and about half of the phage would precisely delete a region 3' of C alpha which included the XX1.3 and XX1.8 region (Fig. 1). For enhancer analysis, DNA fragments from both phage were subcloned into pA10CAT-2 (29) 3' to the CAT gene, using either Xba I or partial digestion with EcoR1. The plasmid pX1.0 is pA10CAT-2 with an Xba I 1.0 kb fragment containing the Ig heavy chain enhancer cloned 3' of CAT (30). The plasmid pSV2CAT has the SV40 enhancer and early promoter region 5' of CAT (31).

Transfection experiments Plasmids were prepared by CsCl gradient

centrifugation followed by extraction of the ethidium bromide with CsCl-saturated isopropanol (40 ml isopropanol:8 ml water: 3.6 g CsCl). Plasmids were then dialyzed for 1 hour against 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA (TE) and treated with 100 micrograms per ml of proteinase K. The plasmids were then extracted twice with phenol/chloroform, once with chloroform, once with ether and dialyzed for 2 days at room temperature against TE with 5 changes of buffer. This purification is necessary for efficient transfection of P3X63-Ag8 using calcium phosphate precipitates. At least two different preparations of plasmid contructions were used in transfection experiments. Transfections, cell extract preparation and CAT assays were performed as described previously (30,31). In the first series described, 6.8 picomoles (the molar equivalent of 30 micrograms of the plasmid pX1.0 ;6.65 kb) were transfected and in the other series, 11.4 picomoles (50 micrograms of pX1.0) were transfected. The CAT activity data were quantitated either by cutting out the radioactive spots of thin layer chromatography plates followed by liquid scintillation counting or by use of the AMBIS beta scanner system (Automated Microbiology Systems, San Diego, CA, USA).

When beta-galactosidase control experiments were performed, 15-50 micrograms of the plasmid pCH110 (32) was cotransfected with the test construct. For extract preparation, the aliquot of cells was divided into two halves one of which was harvested as for CAT assays. The other half was treated and assayed for beta-galactosidase as described (33). The beta-galactosidase control yielded a significant signal above endogenous backround only in the more efficient experiments. Thus, it was not possible to utilize an internal control in experiments of lower efficiency.

To search for enhancer activity specific for the c-myc cryptic promoter region, this region was inserted 5' of CAT. The cryptic promoter/CAT fusion constructions were made by cloning the Xba I 1.65 kb cryptic start region of M603 (15) into the plasmid linker region of pMT3 (a pBR322 derivative). The cryptic start region fragment was then isolated on a Bgl II-HindIII fragment by using the flanking restriction sites in the linker region and substituted for the SV40 early promoter of pA10CAT-2 and pX1.0 to form XX1.65/CAT and XX1.65/CAT-X1.0, respectively. The XX1.65/CAT plasmid was then partially digested with EcoR1 and the two EcoR1 fragments (R1R14.5 kb and R1R19.2 kb derived from Ch3'Cα12A) were subcloned 3' of CAT.

Stable transfections were performed using reconstructions of the translocation juncture in a plasmid vector. For later RNA analysis, the Xba I site just 5' of c-myc exon 2 was digested with Xba I, endfilled using the

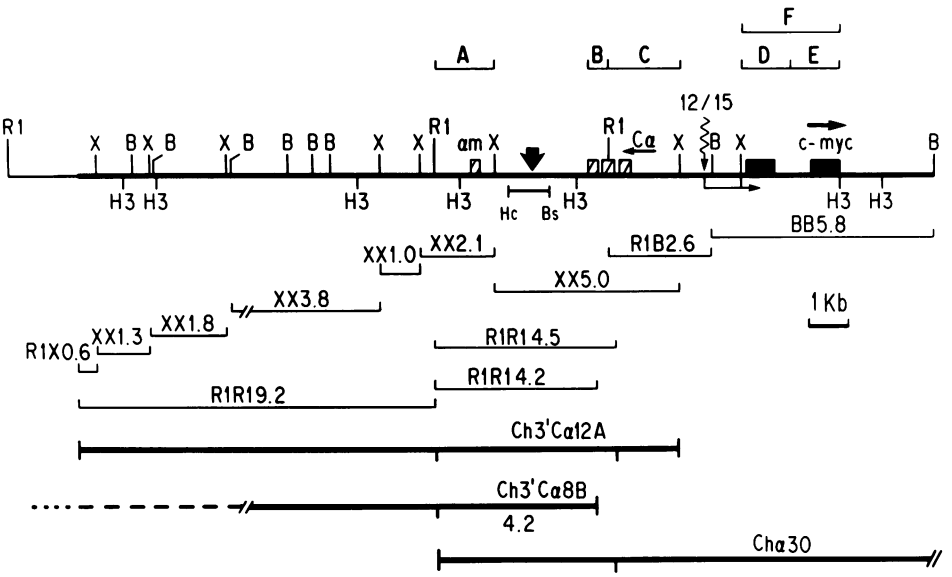

Figure 1. A restriction map of the C alpha/c-myc translocation juncture in plasmacytoma M603 . The two filled boxes at the right are the coding exons 2 and 3 of c-myc; hatched boxes indicate the three exons encoding the C alpha exons. The wavy arrow marked "12/15" indicates the junction site between sequences derived from chromosome 12 and chromosome 15. The horizontal arrows near c-myc and C alpha indicate the normal direction of transcription of the two loci. The open box fused to an arrow 5' of the first c-myc coding exon indicates the region of cryptic start sites (15). The broad dark vertical arrow indicates the position of a DNase I hypersensitive mapped 3' of C alpha (Fig. 4). Hybridization probes used in various experiments are marked above the map: A, EcoRl-Xba I 1.6 kb; B, HindIII-EcoRl 0.7 kb; C, EcoRl-Xba I 1.9 kb; D, Xba I-Sst I 0.9 kb; E, Sst I-HindIII 1.6 kb; F, Xba I-HindIII 2.5 kb. Below the map are indicated the restriction fragments tested for enhancer activity. The bar marked with Hc-Bs is the BsteII-HincII 1.0 kb fragment which contains the DNase I hypersensitive site and which was tested for enhancer activity in series 3 (Table 1). The lowest lines show regions contained phage clones, Ch3'Cα8B and Ch3'Cα12A. The hash marks in the XX3.8 fragment (derived from CH3'Cα8B) and in the CH3'Cα8B phage insert indicate the site of discontinuity of the clones with respect to the genome. The dotted line of Ch3'Cα8B indicates sequence in this clone not found at this site in the genome.The restriction sites are Rl, EcoRl; X, Xba I; B, BamHI; H3, HindIII; Bs, Bste II; Hi, Hinc II. Not all Bste II and Hinc II sites are indicated. Note that in some experiments the RlR14.2 fragment (derived from CH3'Cα8B) was used whereas in others the RlR14.5 fragment (CH3'Cα12A). The two fragments differ by approximately 200 bp in the C alpha coding region.

Klenow fragment of DNA polymerase I and nucleotides, and finally blunt end ligated. The four base pair insertion allowed us to distinguish endogenous from transfected translocated c-myc transcripts in RNA analyses (see below). All constructs contained this mutation and ended 3' of c-myc at the BamHI

Table 1. Summary of Enhancer Search Data

construct	n	%SV2CAT	
Series 1			
pSV2CAT	6	100	
pA10CAT-2	6	0	
pX1.0	6	88	(+/- 23)
BB5.8 A	3	0	
BB5.8 B	3	1.3	(+/- 2.3)
R1B2.6 A	3	0	
R1B2.6 B	3	4.3	(+/- 7.4)
XX5.0 A	3	0	
XX5.0 B	3	0.9	(+/- 1.4)
Series 2			
pSV2CAT	28	100	
pA10CAT-2	25	0.41	(+/- 0.53)
pX1.0	19	23.0	(+/- 10.6)
R1R14.2 A	11	3.7	(+/- 3.2)
R1R14.2 B	11	2.2	(+/- 2.6)
R1R19.2 A	10	0.8	(+/- 1.2)
R1R19.2 B	8	1.5	(+/- 2.6)
XX2.1	3	0	
XX1.0	3	1.1	(+/- 1.3)
XX3.8	3	0	
XX1.8	4	0.64	(+/- 0.28)
XX1.3	3	0.43	(+/- 0.75)
R1X0.6	3	0	
Series 3			
pSV2CAT	3	100	
pA10CAT-2	3	1.7	(+/- 1.2)
Bste-Hinc 3'A	3	2.7	(+/- 1.1)
Bste-Hinc 3'B	3	1.7	(+/- 1.6)
Bste-Hinc 5'A	3	1.7	(+/- 1.4)
Bste-Hinc 5'B	3	1.8	(+/- 0.32)

Enhancer activity in a 22 kb region surrounding the C alpha gene segment. "n" indicates the number of transfection experiments, %SV2CAT indicating the average % CAT activity of the postive control pSV2CAT, + or - the standard deviation. The average conversion of chloramphenicol label to acetylated derivatives for pSV2CAT in the second series of experiments was 18.5% (n= 26) in the 3 hour assay. The pX1.0 construct is pA10CAT-2 with the IgH enhancer X1.0 fragment 3' of CAT. A value of "0" indicates that CAT activity was not detectable with this construct.

site. At the 5' end, we made constructs which ended at the Xba I site just to the left of the juncture (pXB-myc, Fig. 1), the EcoR1 site in the middle of the C alpha coding segments (pRXB-myc) and at the EcoR1 site 4.5 kb further 5' (relative to c-myc;pRRXB-myc). We also made a construct with the 9.2 kb EcoR1 fragment 3' of C alpha cloned into the EcoR1 site in the C alpha coding region of pRXB-myc. The constructs were cotransfected with pSV2neo (34) at a 2 to 1 molar ratio to obtain low copy number insertion. Transfectants were selected with G418 at 500 ug/ml and several stable pools formed after 3-4 weeks. Southern blot analysis of the stable pools demonstrated that 10-30 copies of the

constructs were stably integrated. Expression of the transfected constructs in these cells was analyzed using RNase protection (35) and a RNA probe synthesized from pSp6-4 (35) containing the 406 bp Pst I fragment which covers the cryptic start region just 5' of the mutated Xba I site and ends within the 5' part of c-myc exon 2 (15).

Run-on transcription experiments The run-on transcription experiments were performed as described previously (36). The DNA probes used are indicated in Fig. 1 and in Tables 2 and 3. Between 10 and 80 million cpm were incorporated into RNA in each experiment. Duplicate nitrocellulose dots for each probe were hybridized with labeled RNA, washed and autoradiographed and finally counted by liquid scintillation. The total cpm for each probe were subtracted from the total for the appropiate backround vector (plasmid pMT3 or M13mp18) yielding net counts shown in Tables 2 and 3.

Chromatin structure analysis The analysis of C alpha chromatin was performed with DNase I digestion series prepared previously for c-myc chromatin analysis (23). The analysis is the same as previously described (23) except that the specific restriction digests and hybridization probes are as described in Fig. 4.

RESULTS

No enhancer activity is detected 16 kb 5'of the translocated c-myc gene. The transcriptional activation observed in translocated c-myc genes is similar to that expected for enhancer elements. Since the IgH enhancer is not found near translocated c-myc genes in most cases, we decided to search the region surrounding a translocated c-myc gene to determine if any novel enhancer element could be detected.

In order to search for a transcriptional enhancer, additional DNA sequences 3' of the C alpha gene segment adjacent to the translocated c-myc gene in the tumor M603 were cloned. Figure 1 shows a restriction map of the translocation juncture in the murine plasmacytoma M603, an IgA producer. The alpha 30 lambda phage clone encompassing the juncture was cloned and described previously (10). Two other lambda phage clones, Ch3'C_{α}8B and Ch3'C_{α}12A were obtained by screening a mouse sperm genomic library constructed in Charon 4 using probe B (Fig. 1). The two new clones contain overlapping regions which are identical but Ch8B was found to contain a repetitive region near its 3' end (indicated by a dashed line in Fig. 1) which was not found in the genome at this site. The map shown for CH12A corresponds to genomic DNA for the

region 3' of C alpha on the basis of genomic Southern blots with five different restriction enzymes (data not shown).

To search this region for a transcriptional enhancer, overlapping restriction fragments, indicated in Fig. 1, were cloned into the vector pA10CAT-2 (29) at sites 3' to the chloramphenicol acetyl transferase (CAT) gene. Transcription from the SV40 early promoter is dependent on enhancing activity in the restriction fragments subcloned 3' to CAT. The constructs were transfected into the murine plasmacytoma cell line P3X63-Ag8 (37) and cellular extracts were prepared and assayed for CAT activity 2 days later. In the first series of experiments (Table 1), no significant enhancer activity was found within the region near the translocation juncture, although the SV40 and Ig heavy chain enhancer controls showed considerable activity. In the second series, regions further 3' of C were tested with overlapping subclones and again no strong enhancing activity was observed (Table 1). Some weak activity was observed for the R1R14.2 fragment in both orientations but this activity was variable (Table 1). In the third series, a region within the R1R14.2 fragment which contains a prominent DNase I hypersensitive site in the tumors M104E (Fig. 3), T1017, and M173 (data not shown) was tested for enhancing activity both 5' and 3' to CAT. Again, no enhancing activity was seen (Table 1). Thus, we conclude that no detectable enhancer element is found within the R1R14.2 fragment. In summary, the data show that no enhancer element detectable in this assay is present in the alpha constant or c-myc regions tested.

In our search for a novel enhancer element, we performed control experiments to ensure that non-random variations in transfection efficiency did not distort our results. Cotransfections were performed with a plasmid containing the beta galactosidase gene under control of the SV40 early promoter and enhancer (pCH110, ref. 32). Cotransfection experiments showed that although efficiency did occasionally vary up to a maximum two fold within different plates of a given transfection experiment, the differences were neither consistent nor great enough to account for lack of activity in the different constructs. We conclude that variations in transfection efficiency did not affect our overall conclusion that an enhancer is not detectable in the region tested.

Transcription from the cryptic start region is not enhanced by sequences near C alpha. We considered it possible that an enhancer located near the alpha constant region might not work well with the SV40 early promoter but might specifically activate transcription from the cryptic c-myc start sites used in vivo. To test this possibility, we constructed CAT vectors which substituted

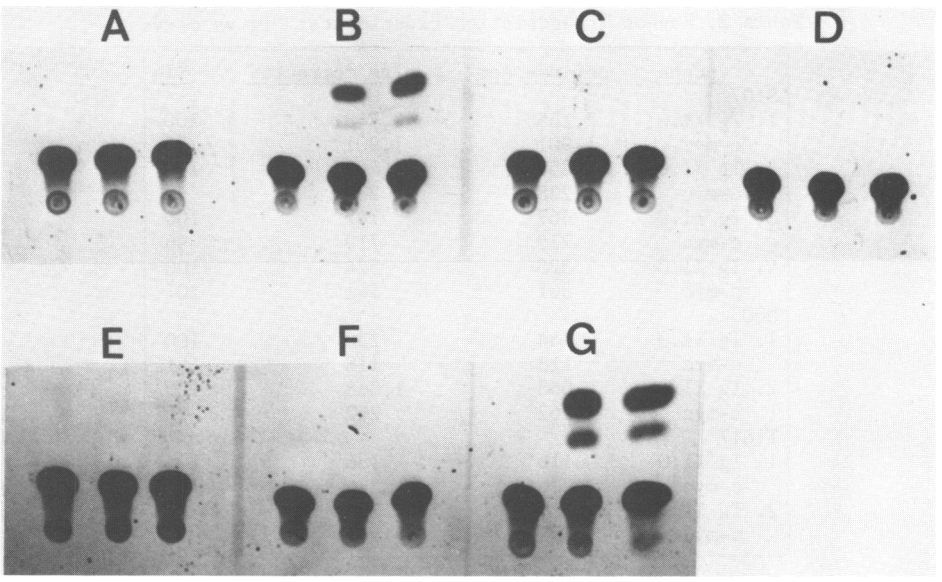

Figure 2. CAT activity from cryptic promoter constructs. Autoradiographs of TLC plates are shown; labeled chloramphenicol is nearest the origin whereas the acetylated derivatives migrate further up the TLC plate. The quantity of acetylated chloramphenicol is a measure of the quantity of transcription for each construct. A set of three time points in the CAT assay, 0, 2 and 3 hours (left to right), are shown for each contruct tested. A. cryptic promoter region 5' of CAT, B. cryptic promoter region with the IgH enhancer on an Xba I 1.0 kb fragment 3' of CAT, C. and D. cryptic promoter region with the R1R1 4.5 kb fragment in different orientations 3' of CAT, E. and F. cryptic promoter region with the R1R19.2 kb fragment in different orientations 3' of CAT, G. SV40 early promoter with the IgH enhancer (pX1.0) 3' of CAT.

a 1.65 kb Xba I fragment encompassing the majority of the cryptic start sites of the M603 translocation juncture (15) for the SV40 early promoter of pA10CAT-2 5' of the CAT gene. The R1R14.5 and R1R19.2 fragments of CH3'C$_\lambda$12A were inserted by partial EcoR1 digestion 3' to the CAT gene. A construct with the Xba 1.0 kb IgH enhancer sequences 3' of CAT was also made as a positive control. When tested by transfection into P3X63-Ag8, we found that the cryptic start site region gave very weak CAT activity (about 10% of pA10CAT-2 and 800-1000 fold less than pSV2CAT). Neither the R1R14.5 nor the R1R19.2 fragments in either orientation enhanced CAT transcription (a minimum of 5 experiments for each construct; Fig. 2). The presence of the IgH enhancer 3' of CAT did stimulate transcription but this activity was about 5-7 fold less than the activity of the IgH enhancer with the SV40 early promoter (Fig. 2). These data show that an enhancer relatively specific for the cryptic start region

Table 2. Run-on Transcription Experiments: Ig vs c-myc

probe	net raw cpm	size corrected	%Ig
S107			
1. Ig X1.0	2,155	3,448	100
c-myc	1,901	1,901	55
2. Ig X1.0	251	402	100
c-myc	238	238	59
3. Ig X1.0	467	747	100
c-myc	719	719	96
4. Ig X1.0	328	524	100
c-myc	561	561	107
M603			
1. Ig X1.0	144	230	100
c-myc	118	118	51
2. Ig X1.0	668	1,068	100
c-myc	262	262	25
T1017			
1. Ig X1.0	116	186	100
c-myc	226	226	122
2. Ig X1.0	139	222	100
c-myc	172	172	77

Run-on transcription experiments comparing polymerase loading on IgH and c-myc genes in plasmacytomas. The rate of transcription of IgH was measured with the Xba 1.0 probe and that of c-myc with probe E in Fig. 1. "Net cpm" indicates the total counts over backround. Column two shows cpm adjusted for probe length. Transcription of c-myc as a percentage of IgH is presented in column 3.

does not exist in the 3' alpha region tested and also that the cryptic start region alone does not contain strong promoters of transcription. Finally, cryptic start sites in the region appear to be activated by the IgH enhancer as has previously been demonstrated by direct analysis of the RNA (21,22). Cryptic c-myc promoters are not active after stable transfection of translocated c-myc constructs. The use of fusion constructions and transient transfection might in some way have interfered with our ability to detect an activating element. In order to test for activating sequences in an intact translocated c-myc gene stably integrated into a chromosome, we made stable transfectants containing reconstructions of the translocation juncture. The constructions contain DNA extending from the BamHI site 3' of c-myc exon 3 (Fig. 1) to the translocation juncture and at the 5' end include different quantities of sequence from the C_{α} locus. A four base pair insertion just 5' of exon 2 allowed us to distinguish endogenous from transfected transcripts using an RNase protection assay (35). Pools of stable transfectants were selected using pSV2neo and G418 (34) and contained 10-30 copies of each construct per cell. Very little if any transcription from the cryptic start

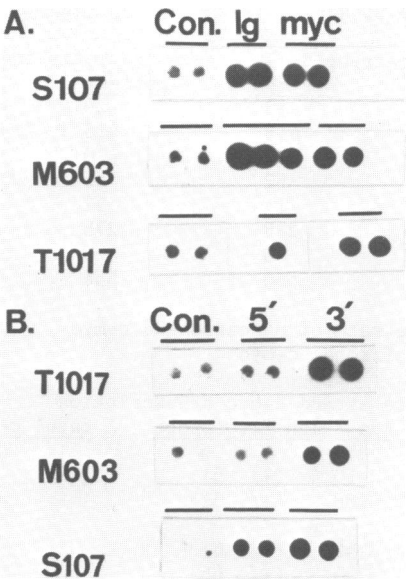

Figure 3. Run-on transcription experiments. Autoradiograms of nitrocellulose dots bound with DNA probes hybridized to specific radiolabeled RNA's are shown. A. A comparison of IgH (Xba 1.0 enhancer region) versus c-myc (Sst I-HindIII fragment, probe E in Fig. 1) for three plasmacytomas is shown. Individual experiments in each plasmacytoma are indicated. The control plasmid is the cloning vector, pMT3 (a pBR322 derivative). B. A comparison of 5' versus 3' sense strand transcription is shown. The 5' probe is a 0.4 kb BamHI-SstI fragment from within c-myc exon 1 cloned into M13mp11 (15). The 3' probe is an M13mp11 clone of the Pst I 0.9 kb fragment of the c-myc cDNA myc54 (12) which covers a small amount of exon 2 and all of exon 3. The control vector is M13mp11. The exposures shown are from 16-30 hours with an intensifying screen.

sites was detectable in any of the transfectants although endogenous c-myc transcripts were easily detected (data not shown). From these experiments, we found no evidence that any region can activate c-myc transcription (data not shown). This result makes it unlikely that a strong cis-acting element that is detectable by stable transfection experiments exists in this region. The translocated c-myc locus is transcribed at high rates comparable to that of the Ig heavy chain locus. Although our previous work showed a striking increase in DNase I sensitivity of the chromatin structure of the translocated c-myc gene (23), which suggested strong transcriptional activation, our finding that there was no enhancer detectable in the region and that the cryptic promoter region alone was not active suggested that the transcription rate of translocated c-myc genes might be low. We decided to perform run-on tran-

Table 3. Run-on Transcription Experiments

Analysis of unrearranged c-myc gene transcription in T1017

c-myc probe	net cpm	corrected	% of total
1. 5' B-S 0.4	13	29	7
3' Pst 0.9	416	416	100
2. 5' B-S 0.4	0	0	0
3' Pst 0.9	52	52	100
3. 5' B-S 0.4	14	32	11
3' Pst 0.9	278	278	100
4. 5' B-S 0.4	0	0	0
3' Pst 0.9	556	556	100

Average relative rate of unrearranged c-myc as a fraction of total c-myc 4.5 (+/- 5.4%)

Analysis of sense strand c-myc transcription

probe	Ave	% IgH	X1.0 probe	% total c-myc	n
M603					
c-myc	10.4	(+/- 5)		27	4
C alpha	188	(+/- 52)			4
S107					
c-myc	42	(+/- 6)			4
C alpha	334	(+/- 65)		53	3

Table 3. Run-on transcription experiments with strand-specific probes. The top half presents individual experiments comparing normal c-myc transcription (the 5' probe is BamHI-Sst I 0.4 kb, see Fig. 3) with total c-myc transcription (the 3' probe Pst I 0.9 fragment, see Fig. 3). The bottom half of the table compares sense transcription of c-myc with IgH and with total c-myc transcription. The data is a summary of experiments with different probes: the single strand probes for c-myc were probes D (XS 0.9), F (XH2.5) or 3'PstO.9 (see Figs.1 and 3) in different experiments. For IgH, all experiments used the Xba 1.0 fragment and probe C (R1X1.9;Fig. 1). The values are shown +/- the standard deviation. The estimate of the percent of total c-myc transcription that was from the sense strand was made by dividing the percent of IgH observed with single strand probes by the average percent of IgH observed with double strand probes; for M603, it is 10%/ 38% and for S107, it is 42%/ 79%. This is possible because control experiments did not demonstrate antisense transcription of the IgH X1.0. region (data not shown).

scription experiments in plasmacytomas to compare translocated c-myc and IgH transcription rates. Such experiments would allow us to assess whether the translocated c-myc gene was strongly activated at the transcriptional level as suggested by the DNase I sensitive chromatin structure (23).

To measure the rates of transcription in the c-myc and IgH loci, run-on transcription was performed in isolated nuclei from 3 plasmacytomas: M603, S107 and T1017 (9). Radiolabeled RNA was hybridized to double stranded probes indicated in Fig. 1. We found that a very high level of transcription was detected with a probe for the third exon region of c-myc; the rate in most experiments was between 50 and 100% of IgH transcription as measured by the IgH enhancer Xba 1.0 fragment in the different plasmacytomas (Fig. 3A; Table 2).

Since both normal and rearranged c-myc alleles exist in these cells, we wished to determine whether the c-myc transcripts were from the translocated allele. In order to determine unequivocally the transcription of the sense strand of each c-myc allele, we used the tumor line T1017 in which there is no reciprocal 5' fragment of translocation so that a 5' exon 1 probe would measure only normal c-myc transcription (ref. 9 and E.K. unpublished observations). A 3' exon probe would detect both normal and translocated c-myc transcripts. Using the 5' and 3' single stranded probes, we found low or undetectable transcription from the 5' region in T1017 while the 3' region showed substantial transcription (Fig. 3B; Table 3). We also tested the other tumors M603 and S107 although the presence of a reciprocal 5' region might complicate our results. Very little 5' transcription was observed for tumor M603 (Fig. 3B) consistent with the DNase I insensitive chromatin of the normal and reciprocally translocated 5' region (23). S107 has substantial 5' transcription (Fig. 3B). The normal c-myc gene of S107 is relatively insensitive to DNase I (23) and so presumably inactive while the reciprocally translocated 5' region, unlike that of M603, was very sensitive to DNase I (data not shown). Although from these experiments, we cannot be certain that c-myc transcription is not occurring from the normal c-myc gene, the inactive chromatin structure of the normal c-myc locus (23) suggests that all transcription is occurring from the translocated c-myc allele. Thus we conclude that the normal c-myc gene is transcribed at low or undetectable levels in these plasmacytomas and that the high level transcription observed with the 3' c-myc probes is from the translocated c-myc locus .

Since previous experiments demonstrate transcription of both DNA strands within the c-myc locus (14,17,38), we expected the transcriptional activation of translocated c-myc genes to be nonspecific with respect to transcriptional direction. To determine how much transcription occurred from the sense strand of c-myc, we compared transcription of c-myc with that of the IgH locus, using single stranded probes. Several probes for myc (XS0.9, Pst 0.9, XH 2.5; Table 3) and probes for the IgH locus, (X1.0 and R1X1.9;Table 3) were used to measure transcription in S107 and M603. We found that transcription of the myc sense strand was about 27% and 53% of that observed for the whole 3' c-myc locus in M603 and S107, respectively (Table 3). The probes for the alpha region (R1X1.9) consistently bound more radio-labeled RNA than the IgH enhancer intervening sequence probe (X1.0). This may be due to the presence of additional rearranged alpha alleles which lack the IgH enhancer region in these cells (23). These data confirm that the rate of antisense transcription of

translocated c-myc is thus equal or greater than sense transcription as has previously been observed in other murine cell lines (38).

Thus transcription of the 3' c-myc region occurs on both strands and the total rate is high when compared with the actively transcribed IgH genes in these cells. Since we are measuring transcription of one allele of c-myc and multiple alleles of IgH (e.g. about 4 copies of the active alpha chromosome in M603, ref. 23; both active and reciprocally translocated alleles in S107, ref. 9), the relative rate of c-myc to IgH is a minimum estimate. Thus the translocated c-myc gene is strongly activated at the transcriptional level by translocation to the IgH locus although this appears to occur without an adjacent strong enhancer or promoter.

The chromatin structure of the complete Ch locus is not activated by the presence of the IgH enhancer. In the mouse immunoglobulin heavy chain locus, the constant region gene segments are spread over a region of 200 kb with the order (5') IgH enhancer, C_{μ} , C_{δ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2a}$, $C_{\gamma 2b}$, C_{ε} , and C_{α} (3') (39). It has been suggested that the known IgH transcriptional enhancer present in the Ch region prior to c-myc translocation could be responsible for a stable activation of the chromatin structure within the whole 200 kb region and subsequently any gene, such as c-myc, which entered the region would also be activated even if the translocation event were to remove the IgH enhancer (1). A number of plasmacytomas are useful in addressing this question because they secrete mu (M104E), delta (T1017) or gamma (HOPC 1, M173) but have c-myc translocations to the alpha constant region (9). Thus, the C_{α} allele 3' of the expressed C_{μ} , C_{δ} or C_{γ} gene remains unrearranged, and a C_{α} allele on another chromosome is translocated. If the above model is correct, both C alleles would be expected to have open DNase I-sensitive chromatin due to previous activation of the entire Ch locus by the IgH enhancer.

Since the unrearranged C allele on the chromosome transcribing mu, delta, or gamma can be distinguished from translocated alleles by Southern blots, we studied the chromatin structure of the C_{α} alleles using DNase I sensitivity. We found that the unrearranged C_{α} alleles of M104E, T1017, M173 (Fig. 4) and HOPC1 (not shown) were relatively insensitive to DNase I while the translocated C_{α} alleles were very sensitive to DNase I. Previous experiments with c-myc probes using these same DNase I-treated DNA preparations (23) indicate that the DNase I sensitivity of rearranged alpha alleles observed in these experiments was the same as the translocated c-myc gene while that of the unrearranged C_{α} alleles was like the normal c-myc gene. We conclude that the Ig enhancer does not activate the complete Ch region as measured by DNaseI

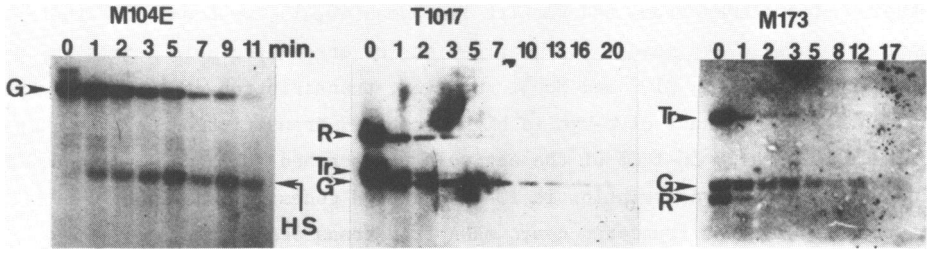

Figure 4. DNase I sensitivity of C alpha alleles in mu, delta, and gamma secreting plasmacytomas. Southern blot analysis of DNase I digestion series are shown. The tumor is indicated above each panel and time of DNase I digestion in minutes is shown above each lane. The "G" indicates the germline unrearranged C alpha allele 3' of the functionally transcribed transcribed Ch gene; "Tr" indicates the C alpha allele translocated to c-myc; "R" indicates another rearranged C alpha allele present in these tumors. The unrearranged C alpha allele was identified by comigration of the bands with the bands of the germline C alpha alleles of liver and spleen on Southern blots. The HS indicates the position of a band representing a DNase I digestion product formed by preferential digestion at a site indicated in Fig. 1. The experiments demonstrating the DNase I hypersensitive site 3' of C alpha in T1017 and M173 are not shown but were performed using an EcoR1 digest and probe A (Fig 1). The digest and probes used for each panel from left to right are: EcoR1, probe B; HindIII, probe C; HindIII, probe C. Note that in the middle panel, the R and Tr alleles appear amplified relative to the G allele. This fact decreases the apparent relative difference in the rate of digestion by DNase I.

sensitivity but cannot exclude from these experiments that activation might occur that is not reflected by the acquisition of DNase I-sensitive chromatin structure.

DISCUSSION

We have sought to understand how cryptic promoters for the c-myc gene are activated after the gene is translocated to the IgH locus in murine plasmacytomas. Although several features of this activation suggested the presence of a transcriptional enhancer element, an exhaustive search for an enhancer revealed no significant enhancer activity in a 22 kb region encompassing the translocated c-myc gene. Furthermore, we found that the c-myc cryptic promoters had low transcription initiation activity in a transient transfection assay. Although the translocated c-myc gene appears to lack a strong promoter or enhancer element, we show it is transcribed at a relatively high rate. Thus, transcriptional activation of translocated c-myc may occur through a novel mechanism.

Transcriptional activation, as well as mRNA stability, contribute to high steady-state levels of c-myc in plasmacytomas. Consistent with previous

studies of a plasmacytoma and Burkitt lymphoma (40,41), we found that the untranslocated c-myc gene is transcribed at low or undetectable levels in plasmacytomas T1017, S107 and M603. However, transcription of both the sense and anti-sense strands of c-myc is high; combined transcription on both strands is usually 50-100% of the actively transcribed IgH genes in these tumors and sense transcription is 10-42 % of IgH genes. It been shown by others (16,42) that truncated c-myc mRNA from translocated genes is more stable than normal c-myc mRNA and it is clear that increased mRNA stability contributes to the increased levels of c-myc mRNA in plasmacytomas. Nevertheless, since there is no evidence of cryptic c-myc transcripts in cells where c-myc is not translocated (15) and since the results presented here show relatively active transcription of c-myc from the cryptic promoters (Table 3), it is clear that transcriptional activation of the translocated c-myc gene is a critical event in determining the deregulated levels of c-myc mRNA in plasma-cytomas.

There is no evidence of enhancer involvement in c-myc cryptic promoter activation. We have carefully analyzed 22 kb of DNA which encompasses the c-myc translocation juncture using both transient and stable transfections and have been unable to find significant enhancing activity which might be responsible for the transcriptional activation of translocated c-myc genes. Stable transfection experiments by others (21) have also been unable to identify an enhancer in a human C alpha/c-myc translocation juncture. There remain a few formal reasons why an enhancer element might have been missed in our studies. An enhancer might be located more than 16 kb from the translocation junction, outside the region analyzed. Also, if the enhancer depended on a cellular protein present in low amounts or absent in the cells we transfected, it would not have been detected. The weak activity in the R1R14.2 fragment (Table 1) could be from such an enhancer dependent on a limiting factor, although failure of this region to activate cryptic promoters in stably transfected lines which had only 10-30 gene copies argues against this possibility. Other enhancers (e.g. SV40 and IgH) do function in the P3X63-Ag8 cell line and this line does contain a translocated c-myc gene and so an enhancer near C alpha should function. It is also possible that a putative enhancer might function best 5' of the promoter rather than 3' as tested, but we doubt that this is a problem since the plasmids are circular and since the functional domain of an enhancer from this region should be very large. Transient and stable transfections similar to those used in this study have been used previously to

identify most known enhancer elements. Thus, we think it likely that c-myc cryptic promoter activation occurs by a novel mechanism which is different from enhancer-dependent activation as it is currently understood.

Although our studies show that a detectable enhancer does not reside near the translocated c-myc gene found in M603, it is clear that heterologous enhancers can activate the c-myc cryptic promoters. In some tumors, the IgH enhancer is near the translocated c-myc gene and may be involved in activation (22,27,28). Transfection (21,22) and transgenic mouse (5,6) experiments have also shown that viral or cellular enhancers can activate c-myc cryptic promoters.

Does the IgH enhancer stably activate the whole Ch locus?

Another suggested explanation for the activation is that the prior presence of the heavy chain enhancer on the same chromosome with the C alpha gene segments 200 kb away might stably activate the Ch locus in B cells (1). Such activation would then be transferred to the incoming c-myc gene. Studies of myelomas with deletions of the IgH enhancer but which still express normal levels of Ig have suggested a stable or permanent activation of the heavy chain locus by the IgH enhancer (43-45). We tested one predicted consequence of this model by studying the chromatin structure of unrearranged alpha alleles in tumors which express other isotypes and which have c-myc translocations with alpha alleles. We assessed the chromatin structure of such alpha alleles using DNase I. Our finding that such alleles have inactive chromatin, similar to that of the inactive normal c-myc gene, argues against the possibility that the chromatin has been activated by the IgH enhancer. This result also suggests that the active chromatin state of c-myc genes translocated to C alpha in tumors such as M104E, T1017, HOPC1 or M173 (23) requires some additional cis-acting process that germline alpha alleles have not undergone which is associated with translocation.

Methylation analyses are also consistent with the notion that the whole Ch locus is not activated by the IgH enhancer since C gene segments far 3' of actively expressed Ch genes are more heavily methylated than rearranged C alpha segments (17, 46,47). Therefore, both methylation and chromatin structure studies suggest that the IgH enhancer does not activate the entire Ch locus.

How is c-myc transcription activated after translocation? Several models could be consistent with the data. A chromatin organizer element might alter the location or structure of the Ch region within the nuclear matrix to allow high level transcription in a manner functionally distinct from enhancer ac-

tivation. Alternatively, activation might be related to the presence of an origin of replication 3' of C alpha (48). Translocated c-myc genes are knwon to replicate early in S phase like Ig genes in B cells while the normal c-myc gene replicates late (48). Matrix attachment sites or origins of replication might not be detectable in transfection experiments assayed for transcription activation.

A better understanding of normal transcriptional activation in the alpha constant region locus may aid our understanding of translocated c-myc activation. Studies of class switching in the I.29 lymphoma line demonstrate the activation of sterile C_{α} transcription of unrearranged C_{α} gene segments when class switching is induced (49,50); similar transcripts have been observed in 18-81 lines which switch isotype (51). There are a number of similarities between unrearranged Ch transcription and translocated c-myc transcription: i) cryptic start site(s) are activated, ii) demethylation of the alpha alleles occurs during switch induction that is similar to that observed for the alpha alleles and c-myc (17,52), iii) c-myc genes are usually rearranged at IgH switch regions consistent with the translocation process occuring in a cell undergoing class switching (9) and iv) in somatic cell hybrids, the transcription of translocated c-myc genes is associated with a mature B cell environment (18-20) similar to that in which switching occurs. Thus, it may be that a novel transcriptional activator not detectable by our transfections which is normally responsible for Ch transcription prior to isotype switching also activates translocated c-myc transcription.

Evidence from other experiments is consistent with the presence of such a novel activator. Studies of spontaneous low-producer variants of MPC 11 suggest that an activator may reside 3' of C gene segments since deletion of a large region of undetermined length 3' of the C_{α} gene segment results in decreased transcription (53). The continued transcription of Ig genes in myelomas which have deleted the IgH enhancer (43-45) could also be explained by such a novel activator functional in mature B-cells. Perhaps experimental protocols utilizing site-directed recombination /mutation in vivo (54), transfection of chromosomes (55), or transgenic mice (56) may succeed in identifying such novel activator sequences.

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