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The c-myc protein represses the λ5 and TdT initiators

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ABSTRACT

The λ5 promoter initiates transcription at multiple sites and confers expression in all cell types. Two λ5 promoter-derived oligonucleotides ($Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$), each with a transcription start site, could promote transcription in transient transfection assays. In contrast, a third oligonucleotide ($+90_{\lambda5}$), without a transcription initiation site, was inactive. The $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$ oligonucleotides formed a major DNA–protein complex B′ in gel retardation analyses; no protein–DNA complexes were observed with the inactive $+90_{\lambda5}$ oligonucleotide. The B′ complexes of $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$ each contained c-myc and myn (murine homologue of Max) proteins. The c-myc and myn proteins were also found to bind the TdT initiator (Inr_{TdT}). Using mutated oligonucleotides, we found that the c-myc/myn proteins bound to the transcription initiation site of both $Inr_{\lambda5:1}$ and Inr_{TdT}, however, these mutated oligonucleotides were inactive in transfection assays. This suggested that, in this system, transcription depended both on a transcription initiation site and appropriate flanking sequences. The significance of c-myc binding to the respective initiator was analysed by overexpressing c-myc in co-transfection assays. Under these conditions the transcriptional activity of both the λ5 and the TdT initiator was repressed.

INTRODUCTION

The λ5 gene is expressed only in pre-B cells; no other cells of the B lineage, or any other cell types tested, express the gene (1, 2). This pre-B cell-specific expression is regulated at the level of transcription (3, 4). The promoter of λ5 is TATA-less, non-GC-rich, and initiates transcription at multiple sites (3, 4). We have previously shown (4) that the region just 5′ of the λ5 gene can be divided into 2 regions, $A_{\lambda5}$ (position –45 to +109) and $B_{\lambda5}$ (position –613 to –45). The $A_{\lambda5}$ region contains all the transcription initiation sites and, in agreement with this, acted as the promoter. The $A_{\lambda5}$ promoter was active in all cell types tested in the presence of a heterologous enhancer. $B_{\lambda5}$ suppressed expression driven by the $A_{\lambda5}$ promoter in non-pre-B cells, thus explaining the pre-B cell-specific expression. Furthermore, $B_{\lambda5}$ acted as an enhancer on a heterologous promoter.

The $A_{\lambda5}$ promoter is very similar to the TdT (terminal deoxynucleotidyl transferase) promoter, in that it shares stretches of sequence homology in the 5′ region, is TATA-less and non-GC-rich (5, 6). A so-called initiator (Inr) has been defined as the TdT promoter element (5). It contains the transcription start site, allows formation of a transcription initiation complex, and confers transcription of a reporter gene *in vitro* and in transient transfection assays. Initiators have also been described for several other promoters with or without a TATA box (7). Previous analyses of the $A_{\lambda5}$ promoter suggested that it might also behave as an initiator (4).

It has been suggested that initiators recruit the TATA binding protein and the general transcription machinery through initiator binding proteins (IBPs) (8). Among the IBPs are TFII-I, USF (upstream stimulatory factor), YY1 (yin-yang-1) and E2F (9–12). It has also been reported that myc, in the presence of TFII-I, can bind to the viral AdML (adenovirus major late) initiator (13).

MATERIALS AND METHODS

Tissue culture conditions

The murine pre-B cell line 230-238 (14) was cultured in Iscove's modified Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, antibiotics and 5×10^{-5} M 2-mercaptoethanol.

Transfections, CAT and luciferase assays

Cells were transiently transfected using DEAE–dextran as described (4). In the co-transfections the ratio of test construct to control (pCMV-luciferase, see below) was 7:1. In some co-transfections 5 μg of various myc expression vectors (see below) were included. Cells were harvested 40–46 h after transfection. Cell extracts were prepared, tested in CAT assays as described (4) and in luciferase assays as described (15). The thin-layer chromatography plates (CAT assay) were measured on a phosphorimager (Compaq; Molecular Dynamics, Sunnyvale, CA). The amount of CAT activity was determined as the amount of acetylated chloramphenicol as a percentage of the total amount of radioactivity in the respective sample. The CAT activities were adjusted relative to the internal control.

DNA constructs

All DNA constructs were made using conventional DNA techniques (16). The pCMV-luciferase construct (17) was a kind gift from Dr U. Deuschle. The HSR-c-myc (human c-myc) and the Δ371 (human c-myc with deletion of the HLH region)

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constructs (18) were a kind gift from Dr W. Lee. The myc and max fusion vectors for expression in bacteria (19) were a kind gift from Dr R. Eisenman. The +90λ_5-CAT-E_μ transfection vector has been described before (4). The Inr$\lambda_{5:1}$-, Inr$\lambda_{5:2}$-, Inr$\lambda_{5:90}$-, Inr$\lambda_{5:SV}$-, Inr$_{TdT}$- and Inr$_{TdT:SV}$-CAT-E_μ were made by cloning oligonucleotides corresponding to the respective sequence (see below) into the unique *Hin*dIII or between the *Hin*dIII and *Sph*I sites 5′ of the CAT gene in the pUC-CAT-E_μ vector (20). The promoter constructs were sequenced according to the manufacturer's suggestions (Sequenase; United States Biochemical, Cleveland, OH).

Nuclear extracts

Nuclear extracts were prepared as described previously (21). The protein concentration was determined according to Bradford (22).

Bacterial extracts

The myc and max expression vectors (see DNA constructs) were freshly transformed into bacteria. Expression of fusion proteins was induced by addition of 1 mM IPTG (19). The kinetics of induction were analysed by SDS–PAGE. Optimal induction was reached after 2–4 h. Bacterial extracts were prepared as 'whole cell extracts' as described (21). The myc and max fusion proteins were shown to be recognised by the anti-myc and anti-Max polyclonal antibodies by Western blot analysis (unpublished results).

Oligonucleotides

The following oligonucleotides were used in this report:

Inr$\lambda_{5:1}$	5′ TACACAGATCCACCTGCACTGGAA
Inr$\lambda_{5:2}$	5′ GGAGATCTACACTGCAAGTGAGGCTAGAGTT
+90λ_5	5′ GACTTTGGACTTGAGGGTCA
Inr$\lambda_{5:90}$	5′ GACTTTACACAGATCGGTCA
Inr$\lambda_{5:SV}$	5′ GACTTTACACAGATCGGTCA
Inr$_{TDT}$	5′ GCCCTCATTCTGGAGAC
Inr$_{TDT:SV}$	5′ CTCCTCTCATTCTGGAATA
DHFRI	5′ GGCGCGACACCCACGTGCCCTCTCT
DHFRII	5′ GCCCCACCTGGCCCCGCCCGGTTGA

Inr$\lambda_{5:2}$, Inr$\lambda_{5:90}$, Inr$\lambda_{5:SV}$, Inr$_{TdT}$, Inr$_{TDT:SV}$ and +90λ_5 contain a *Hin*dIII site (AGCTT) at the 5′ end and a *Sph*I site (GCATG) at the 3′ end.

The following mutant oligonucleotides with mutations in the E-box element of Inr$\lambda_{5:1}$ were used:

mInr$\lambda_{5:1}$	5′ TACACAGATC**CgCCTG**CACTGGAA
mAInr$\lambda_{5:1}$	5′ TACACAGATC**CACCTa**CACTGGAA
mATInr$\lambda_{5:1}$	5′ TACACAGATC**tACCTa**CACTGGAA

Gel retardation assays

Gel retardation assays were performed as described (21). A phosphorimager (Compaq; Molecular Dynamics, Sunnyvale, CA) was used for quantitation of the DNA–protein interactions.

Competition assays

For *in vitro* competition assays, gel retardation reactions were incubated with increasing molar concentrations (see text) of the unlabelled oligonucleotides for 30 min at room temperature prior to the addition of the ^{32}P-end-labelled oligonucleotide. The incubation was continued for 30 min.

Antibodies and peptides

The antibodies and peptides used in this study were: a rabbit polyclonal anti-c-Myc antiserum recognising human and mouse c-myc proteins (Upstate Biotechnology Inc., NY); a monoclonal anti-c-Myc antibody, 9E10 (Cambridge Research Biochemicals, UK), originally shown to recognise only human c-myc (23) (we have found that the 9E10 monoclonal antibody also recognises murine c-myc by Western blot analysis using a more sensitive detection system, ECL, enhanced chemiluminescence; Amersham, UK; unpublished results); the 9E10 c-Myc peptide used as antigen (A-E-E-Q-K-L-I-S-E-E-D-L-L-R-K-R-R-E-Q-L-K-H-K-L-E-Q-L-R-N-S-C; Cambridge Research Biochemicals, UK); supernatants of the hybridomas, clones 9E10.2 and CT14 (ATCC, USA), producing anti-c-Myc antibodies recognising human and mouse c-myc proteins, dialysed against gel retardation buffer; sheep polyclonal anti-c-fos antiserum recognising mouse and human c-fos proteins (Cambridge Research Biochemicals, UK); polyclonal anti-Max (Max-I, Max-II, Max-III) and monoclonal anti-Max (M2.6) antibodies recognising human Max and mouse myn proteins (generously provided by Dr A. Wenzel, Heidelberg, Germany); rabbit polyclonal anti-USF antiserum (C20) recognising mouse and human USF proteins (Santa Cruz Biotechnology Inc., CA); rabbit polyclonal anti-Mxi-1 (C17) antiserum recognising mammalian Mxi-1 proteins (Santa Cruz Biotechnology Inc., CA); and rabbit polyclonal anti-Mad (C19) antiserum recognising mammalian Mad proteins (Santa Cruz Biotechnology Inc., CA). The concentration of each antibody used is indicated in the text.

RESULTS

Initiator-like activity in a minimal λ5 promoter

We first asked whether each of the Aλ_5 start sites could function in isolation. To test this, we inserted such Aλ_5 sequences upstream of the CAT reporter gene in the presence of the heterologous E_μ enhancer, which is required for measurable reporter gene activity (4, 24). The Aλ_5 regions analysed correspond to position –5 to +20, (–5/+20)λ_5-CAT-E_μ, containing two start sites (nos 3 and 4), position +59 to +89, (+59/+89)λ_5-CAT-E_μ, containing one initiation site (no. 7), and position +90 to +109, (+90/+109)λ_5-CAT-E_μ, lacking a start site and shown previously (4) to be inactive (Figs 1 and 2A). These constructs were transiently transfected into pre-B cells together with pCMV-luciferase as control for transfection efficiency. As shown in Figure 2B, both the (–5/+20)λ_5-CAT-E_μ and the (+59/+89)λ_5-CAT-E_μ constructs were transcriptionally active. Their activities were 10-fold above the background activity of either (+90/+109)λ_5-CAT-E_μ or vector (CAT-E_μ) alone (Fig. 2B and data not shown). Thus 25–30 bp of the λ5 promoter were enough to confer transcriptional activity on a reporter gene provided that a transcription start site was present. These data suggested that the

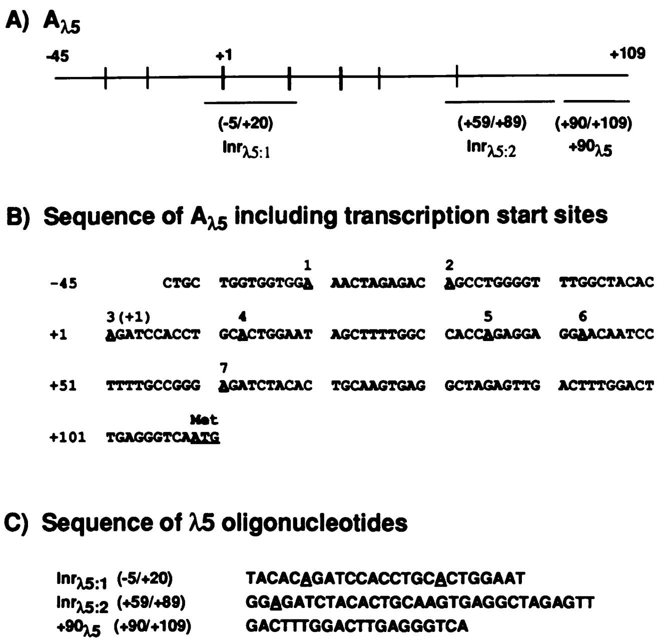


Figure 1. (**A**) The $A_{\lambda5}$ basal promoter. Depicted is the $A_{\lambda5}$ basal promoter (position –45 to +109) including transcription initiation sites (the most 5′ major start site is set as +1). The thin and thick bars represent minor and major start sites, respectively. The lines below indicate the parts of $A_{\lambda5}$ used in different assays. Numbers correspond to nucleotide positions within the promoter. (**B**) Sequence of $A_{\lambda5}$. Shown is the sequence of the basal $A_{\lambda5}$ promoter (position –45 to +109) including transcription initiation sites nos 1–7 (underlined). The most 5′ major start site, no. 3, was set as +1. (**C**) Sequence of λ5 oligonucleotides. Sequence of the λ5 oligonucleotides used in transfection experiments and in gel retardation assays are shown (Materials and Methods). The oligonucleotides $Inr_{\lambda5:1}$ (position –5 to +20), $Inr_{\lambda5:2}$ (position +59 to +89) and $+90_{\lambda5}$ (position +90 to +109) are all derived from the $A_{\lambda5}$ promoter (for locations see above). Transcription start sites are underlined.

λ5 basal promoter consists of several transcription initiation sites that could act independently as initiator-like elements.

A major DNA–protein complex formed with $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$

We next used gel retardation analyses to ask whether proteins present in nuclear extracts could interact specifically with the λ5 sequences analysed above. We defined position –5 to +20, $Inr_{\lambda5:1}$, position +59 to +89, $Inr_{\lambda5:2}$, and position +90 to +109, $+90_{\lambda5}$ (Fig. 1). As shown in Figure 3A, one major DNA–protein complex formed with nuclear extract from 230-238 pre-B cells using either ^{32}P-end-labelled oligonucleotide $Inr_{\lambda5:1}$ or $Inr_{\lambda5:2}$. Furthermore, this complex showed a similar mobility with both oligonucleotides $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$. Minor complexes of higher mobility were also detected following longer exposure times (data not shown). Similar protein interactions were found in both lymphoid and non-lymphoid cell lines (data not shown). It is noteworthy that no protein complexes were found to interact with the ^{32}P-end-labelled oligonucleotide $+90_{\lambda5}$ (Fig. 3A). We concluded that $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$ both formed major DNA–protein complexes migrating with the same mobility.

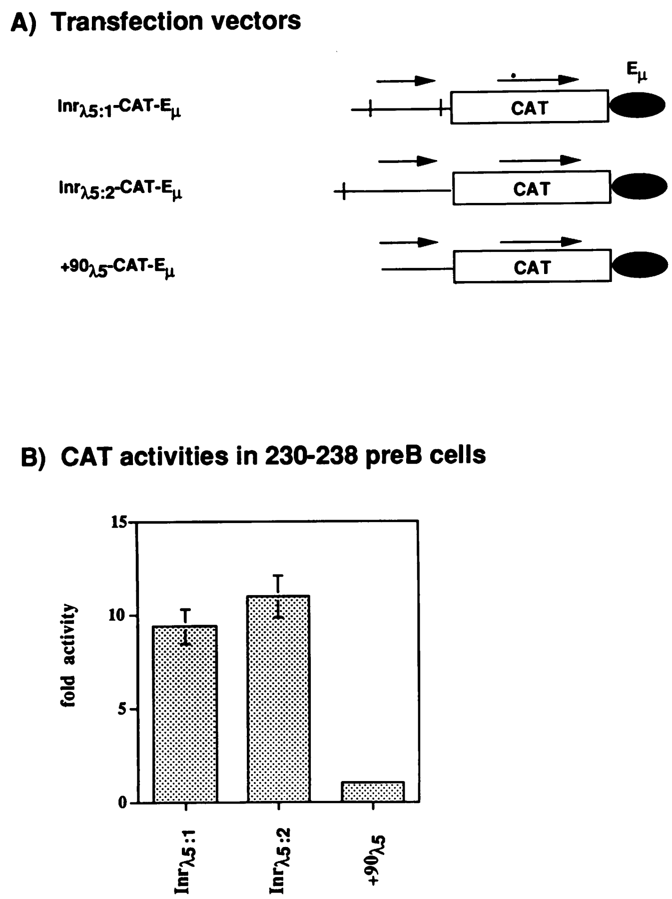


Figure 2. (**A**) Transfection vectors. The transfection vectors contain oligonucleotides corresponding to parts of $A_{\lambda5}$ (as shown in Fig. 1) inserted 5′ of the CAT gene including the E_{μ} enhancer 3′ of CAT. Bars represent transcription start sites. Arrows indicate the transcriptional orientation. (**B**) CAT activity in 230-238 pre-B cells. The figure shows a representative experiment with 230-238 cells transiently transfected with the vectors in (A). The construct $+90_{\lambda5}$-CAT-E_{μ} showed the same activity as vector (CAT-E_{μ}) alone (data not shown). The CAT activities detected with all other constructs were normalised to the $+90_{\lambda5}$-CAT-E_{μ} (set to 1, actual CAT activity 0.5%). All samples were corrected for luciferase activity (internal control).

$Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$ interact with common nuclear protein(s)

The only sequence motif that seemed to be common to both $Inr_{\lambda5:1}$ and $Inr_{\lambda5:2}$ (and lacking in $+90_{\lambda5}$) was an E-box (25), CANNTG, element (Fig. 1C). The E-box motif present in the $Inr_{\lambda5:1}$ sequence is CACCTG, the very same E-box that is found in addition to a CACGTG sequence element in the 5′ flanking region of the DHFR genes (21). These observations prompted us to test if similar DNA–protein complexes would form with ^{32}P-end-labelled oligonucleotides $Inr_{\lambda5:1}$ and DHFRI (CACGTG) or DHFRII (CACCTG). A major complex migrated at the same position with the $Inr_{\lambda5:1}$, $Inr_{\lambda5:2}$ and the DHFR oligonucleotides (Fig. 3A, and data not shown), although it showed different intensities depending on the oligonucleotide used.

The data above raised the question whether there were common proteins in the complexes bound to $Inr_{\lambda5:1}$, $Inr_{\lambda5:2}$ and the DHFR

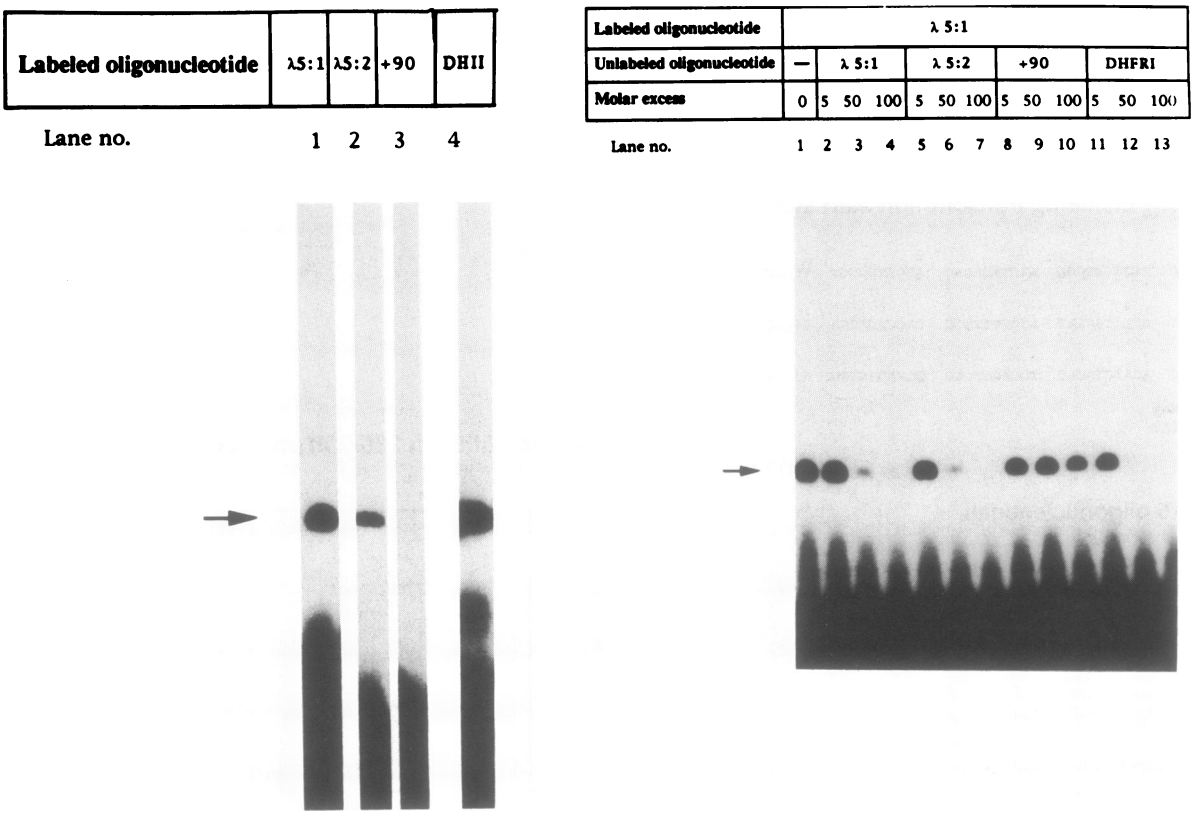


Figure 3. (**A**) Gel retardation analysis of oligonucleotides derived from the $A\lambda_5$ promoter and DHFR 5′ flanking region. 2.5 μg nuclear extract from 230-238 pre-B cells were incubated with ^{32}P-end-labelled oligonucleotides $Inr\lambda_{5:1}$ (lane 1), $Inr\lambda_{5:2}$ (lane 2), $+90\lambda_5$ (lane 3) or DHFRII (lane 4) and analysed on a 5% native polyacrylamide gel (Materials and Methods). The major DNA–protein complex is shown by an arrow. (**B**) *In vitro* competition experiments performed with the $Inr\lambda_{5:1}$ oligonucleotide. 2.5 μg nuclear extract from 230-238 pre-B cells were incubated with ^{32}P-end-labelled oligonucleotide $Inr\lambda_{5:1}$ (lanes 1–13) in the absence (lane 1) or presence of unlabelled oligonucleotides $Inr\lambda_{5:1}$ (lanes 2–4), $Inr\lambda_{5:2}$ (lanes 5–7), $+90\lambda_5$ (lanes 8–10) or DHFRI (lanes 11–13). For sequences see Figure 1C and Materials and Methods. As indicated in the figure, 5–100-fold molar excess of unlabelled oligonucleotides over the ^{32}P-end-labelled $Inr\lambda_{5:1}$ oligonucleotide were used. The major DNA–protein complex is shown by an arrow.

oligonucleotides. We therefore analysed the binding specificity of 230-238 pre-B cell extracts with these oligonucleotides by *in vitro* competition experiments, including $+90\lambda_5$ as a control. As shown in Figure 3B, unlabelled $Inr\lambda_{5:1}$ (lanes 2–4) or $Inr\lambda_{5:2}$ (lanes 5–7) competed equally well with ^{32}P-end-labelled $Inr\lambda_{5:1}$ for the formation of the major complex. Oligonucleotide $+90\lambda_5$ (lanes 8–10) did not interfere with the formation of this complex, in agreement with the lack of protein binding to the $+90\lambda_5$ oligonucleotide (Fig. 3A). DHFRI (lanes 11–13) competed at least as well as $Inr\lambda_{5:1}$ itself. Also, DHFRII competed efficiently with $Inr\lambda_{5:1}$ for the formation of the major complex (data not shown). The same set of *in vitro* competition experiments was performed with ^{32}P-end-labelled oligonucleotide $Inr\lambda_{5:2}$ and led to identical results (data not shown). We concluded that the same nuclear protein(s) were involved in formation of the major complex with the $Inr\lambda_{5:1}$, $Inr\lambda_{5:2}$ and DHFR oligonucleotides.

c-myc and myn bind to $Inr\lambda_{5:1}$ and $Inr\lambda_{5:2}$

A complex, designated B, containing c-myc–Max heterodimers, forms with CACGTG and CACCTG E-box elements in DHFR genes (21, 26). We therefore asked whether the B-like complex formed with the $Inr\lambda_{5:1}$ and $Inr\lambda_{5:2}$ oligonucleotides also contained c-myc and myn (murine homologue of Max). If so, addition of anti-c-Myc or anti-Max antibodies to gel retardation reactions would result in the disruption or supershift of this complex. As shown in Figure 4, using oligonucleotide $Inr\lambda_{5:1}$ as probe, two monoclonal anti-c-Myc antibodies, CT14 and 9E10, disrupted the formation of the complex (lanes 3 and 4). This effect was reversed upon addition of the antigen, the 9E10 peptide, recognised by the 9E10 monoclonal antibody (lane 5). A polyclonal anti-myc antisera supershifted the major complex (data not shown). The addition of three different polyclonal anti-Max antibodies (Max-I, Max-II, Max-III, lanes 6–8) supershifted the B-like complex. A monoclonal anti-Max antibody (M2.6) disrupted the complex (data not shown). The B-like complex was not affected by the addition of either anti-c-fos (lane 2), anti-USF, anti-Mxi-1 or anti-Mad antibodies (data not shown). ^{32}P-end-labelled oligonucleotide $Inr\lambda_{5:2}$ gave identical results (data not shown). We concluded that c-myc and myn proteins could bind to the $Inr\lambda_{5:1}$ and $Inr\lambda_{5:2}$ oligonucleotides. We have therefore provisionally called this $Inr\lambda_{5:1}$ and $Inr\lambda_{5:2}$ DNA–protein complex B′.

c-myc/myn can bind to the initiation site in $Inr\lambda_{5:1}$

Footprinting analysis of the λ5 promoter (6) showed protein binding over the CACCTG E-box and the transcription initiation site present in $Inr\lambda_{5:1}$. Based on this finding and the data above we asked if mutations of the E-box motif in the $Inr\lambda_{5:1}$ sequence

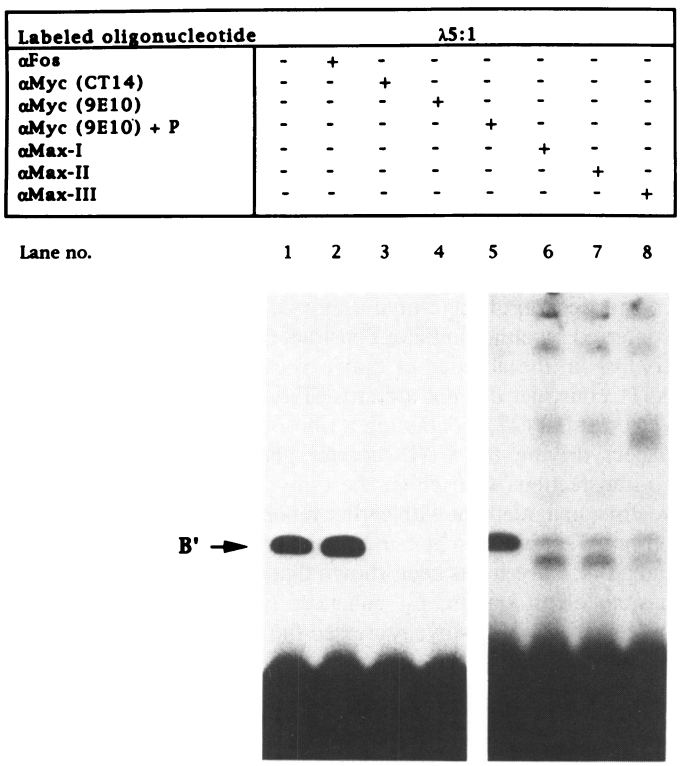


Figure 4. Gel retardation analysis of $Inr_{\lambda 5:1}$ in the absence or presence of antibodies. 2.5 μg nuclear extract from 230-238 pre-B cells were incubated with ^{32}P-end-labelled oligonucleotide $Inr_{\lambda 5:1}$ (lanes 1–8) in either the absence (lane 1) or presence of anti-c-fos (lane 2), anti-c-Myc (CT14 and 9E10) (lanes 3 and 4, respectively), anti-c-Myc (9E10) in conjunction with its specific peptide (lane 5) or anti-Max I–III (lanes 6–8, respectively) antibodies (see Materials and Methods). The following concentrations of antibodies and peptide were used per reaction: anti-c-fos, 100 ng; anti-c-Myc antibodies, 1 μl of dialysed supernatant; polyclonal anti-Max I–III antibodies, 1 μl anti-sera; 9E10 peptide, 100 ng. Complex B′ is shown by an arrow.

interfered with the binding of c-myc and myn proteins. The $Inr_{\lambda 5:1}$ mutants contained single and double point mutations within the CACCTG binding motif (see Materials and Methods). With all the mutants we observed a complex migrating at the same position as complex B′. Furthermore, this complex could be specifically supershifted with antibodies directed against c-myc and Max (data not shown). This suggested either that the introduced mutations did not affect the binding of c-myc/myn to $Inr_{\lambda 5:1}$ or that alternative c-myc/myn binding sites were present within the $Inr_{\lambda 5:1}$ sequence.

As mentioned above, a footprint has been shown over the λ5 transcription initiation site (6) which is part of $Inr_{\lambda 5:1}$. It has also been reported that myc, in the presence of TFII-I, can bind to the AdML initiator (13). We therefore reasoned that c-myc might associate with the initiation site of $Inr_{\lambda 5:1}$; this could explain our results with the $Inr_{\lambda 5:1}$ E-box mutants. To test this, we took advantage of $+90_{\lambda 5}$, which lacked both transcriptional activity and protein binding (Figs 2 and 3). We created $Inr_{\lambda 5:90}$ (Fig. 5A) by inserting position –5 to +5, including start site no. 3 of the $Inr_{\lambda 5:1}$ sequence, into the $+90_{\lambda 5}$ sequence. As shown in Figure 6A, $Inr_{\lambda 5:90}$ formed a major DNA–protein complex. In addition, this complex could be supershifted with anti-c-Myc and anti-Max antibodies but not with anti-c-fos or anti-USF antibodies. To exclude the effect of flanking sequences, we placed the same $Inr_{\lambda 5:1}$ element, position –5 to +5, into another genomic context, SV40 (simian virus 40), thus creating $Inr_{\lambda 5:SV}$. This oligonucleotide retarded a complex co-migrating with complex B′. The $Inr_{\lambda 5:SV}$ complex was also found to contain both c-myc and myn proteins (data not shown). We concluded that c-myc and myn proteins could bind to the $Inr_{\lambda 5:1}$ position –5 to +5, i.e. over the transcription initiation site.

c-myc/myn bind to the initiation site in the TdT initiator

We now asked if c-myc and myn could bind to another initiator. We therefore tested the TdT initiator (Fig. 5A, Inr_{TdT}, position –6 to +11) in our gel retardation assay. As shown in Figure 6B, a major complex formed with the Inr_{TdT} oligonucleotide. Additional minor complexes were also detected after longer exposure times (data not shown). The major complex co-migrated with $Inr_{\lambda 5:1}$ complex B′ and competed with $Inr_{\lambda 5:1}$ for its formation (data not shown). Furthermore, it could be supershifted with antibodies directed against c-myc and Max but not USF (Fig. 6B). To test if the c-myc/myn proteins bound over the transcription initiation site here also, a minimal Inr_{TdT} element (position –3 to +6) was placed into a new genomic context, SV40, thereby creating $Inr_{TdT:SV}$ (Fig. 5A). A B′-like complex, containing c-myc and max proteins, was present also with this newly created oligonucleotide (data not shown). We concluded that c-myc and myn proteins could bind to the Inr_{TdT} position –3 to +6, i.e. over the transcription initiation site.

Bacterially expressed myc and max bind to the λ5 and TdT initiators

To confirm that c-myc and myn proteins bound to the λ5 and TdT initiators, we expressed the myc and max proteins in bacteria (19) and analysed the corresponding extracts in gel retardation assays. Bacterially expressed myc alone and max alone bound to the $Inr_{\lambda 5:1}$, $Inr_{\lambda 5:SV}$, Inr_{TdT} and $Inr_{TdT:SV}$ oligonucleotides but not to $+90_{\lambda 5}$; no binding was observed with bacterial extracts lacking the myc or max fusion proteins. The combination of myc and max extracts showed the same binding patterns (Fig. 7A, and data not shown). Furthermore, polyclonal antibodies directed against either myc or max supershifted the complex, while polyclonal anti-USF antibodies did not (Fig. 7B, and data not shown). We concluded that bacterially expressed myc and max also bound the λ5 and TdT initiators.

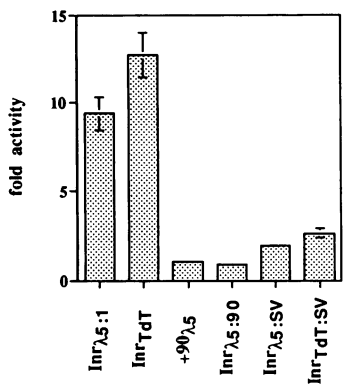
Minimal λ5 and TdT elements lack activity in transient transfection assays

The minimal λ5 and TdT oligonucleotides each contained a transcription start site and bound c-myc/myn, while $+90_{\lambda 5}$ lacked an initiation site and did not bind c-myc/myn. We now asked if the minimal λ5 and TdT initiator sequences conferred transcriptional activity in transient transfection assays. Only low CAT activities were detected with the $Inr_{TdT:SV}$-, $Inr_{\lambda 5:90}$- and $Inr_{\lambda 5:SV}$-containing constructs, while the $Inr_{\lambda 5:1}$-CAT-E_{μ} and Inr_{TdT}-CAT-E_{μ} constructs showed expression levels 10-fold above background (Fig. 5A and B). We concluded that although the minimal λ5 and TdT sequences included a transcription start site and bound c-myc and myn proteins, this was not sufficient to promote high levels of transcription in these assays unless these minimal sequences were flanked by their wild-type nucleotides.

A) Sequence of λ5 and TdT oligonucleotides

$Inr_{\lambda 5:1}$ (-5/+20)	TACACAGATCCACCTGCACTGGAAT
$Inr_{\lambda 5:90}$ (-5/+5)	GACTTTACACAGATCGGTCA
$Inr_{\lambda 5:SV}$ (-5/+5)	CTCCTTACACAGATCGAATA
Inr_{TDT} (-6/+11)	GCCCTCATTCTCGAGAC
$Inr_{TDT:SV}$ (-3/+6)	CTCCTCTCATTCTCGAATA

B) Low activity by minimal λ5 and TdT initiators



C) c-myc represses λ5 and TdT initiators

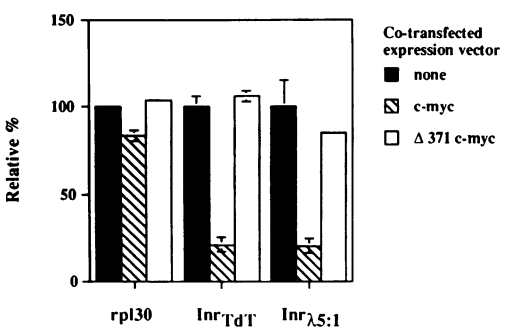


Figure 5. (**A**) Sequence of λ5 and TdT oligonucleotides. Sequence of oligonucleotides used in transfection experiments and in gel retardation assays. $Inr_{\lambda 5:90}$, $Inr_{\lambda 5:1}$ from position –5 to +5 inserted into $+90_{\lambda 5}$; $Inr_{\lambda 5:SV}$, $Inr_{\lambda 5:1}$ from position –5 to +5 inserted into SV40 sequences. Inr_{TdT} corresponds to position –6 to +11 in the TdT promoter (5). $Inr_{TdT:SV}$ corresponds to Inr_{TdT} position –3 to +6 inserted into SV40 sequences. Transcription start sites are underlined. (**B**) Low CAT activity conferred by minimal λ5 and TdT initiators. The oligonucleotides in Fig. 6A were inserted 5′ of the CAT gene, including the E_μ enhancer 3′ of CAT as in Figure 2. The figure shows a representative experiment of 230-238 cells transiently transfected with these vectors. The CAT activities detected were normalised to the $+90_{\lambda 5}$-CAT-E_μ vector (set to 1). All samples were corrected for luciferase activity (internal control). (**C**) Overexpression of c-myc represses the activity of λ5 and TdT initiators. The figure shows a representative experiment of 230-238 cells transiently transfected with the $Inr_{\lambda 5:1}$ or the Inr_{TdT} vectors in (A) or the rpl30 construct which contains the ribosomal protein promoter/enhancer upstream of the CAT gene. The CAT reporter gene vectors were co-transfected with no expression vector (none), with a vector expressing human c-myc (c-myc) or a vector expressing a mutated human c-myc (Δ371). The activity of the respective reporter gene vector, in the absence of any myc expression vector, is set as 100%. For description of vectors, see Materials and Methods.

c-myc down-regulates the expression conferred by the λ5 and TdT initiators

Since the pre-B cell line used above expressed endogenous c-myc, we asked if overexpression of the oncoprotein would affect the activity of the λ5 and TdT initiators. The pre-B cells were therefore transfected with the $Inr_{\lambda 5:1}$-CAT-E_μ and Inr_{TdT}-CAT-E_μ vectors alone or co-transfected with a vector expressing human c-myc (c-myc) or a mutant human c-myc (Δ371) with a deleted HLH region (18). As shown in figure 5C, co-transfection of the c-myc vector down-regulated the activity conferred by either initiator construct 5-fold as compared to the control (in the absence of c-myc vector). The presence of the Δ371 construct did not affect CAT expression levels. Another vector, rpl30 (27), containing a ribosomal protein promoter/enhancer driving the CAT reporter gene, was not affected in co-transfections with either the c-myc or the Δ371 expression vectors, in agreement with earlier reports (28). We attributed the observed suppression by c-myc to the presence of the λ5 and TdT initiators, since it has been shown that overexpression of c-myc has no effect on the E_μ enhancer (28). We concluded that overexpression of c-myc repressed the activity conferred by the λ5 and TdT initiators.

DISCUSSION

This study shows that the analysed initiators ($Inr_{\lambda 5:1}$, $Inr_{\lambda 5:2}$, Inr_{TdT}) are transcriptionally active in the presence of a heterologous enhancer. They all contain: (i) a transcription start site; (ii) have appropriate flanking sequences; and (iii) bind c-myc and myn proteins. The experiments suggested that a transcription start site and appropriate flanking sequences were important for transcription *per se*. The role of c-myc binding to the initiators under normal conditions is not clear, however, overexpression of c-myc in co-transfection experiments specifically repressed reporter gene expression driven off the λ5 and TdT initiators.

We have analysed several λ5 and TdT promoter-derived sequences for transcriptional potential by introducing them into a vector containing the CAT reporter gene with the E_μ enhancer (24) as activating element. The constructs were transiently transfected into a pre-B cell line and the corresponding CAT activities used as a measure of the transcriptional activity of the respective sequence. In this system, two different λ5-derived oligonucleotides ($Inr_{\lambda 5:1}$ and $Inr_{\lambda 5:2}$) acted independently as initiators, increasing the CAT activity at least 10-fold as compared to background activities. Also, the TdT initiator (5) promoted CAT expression levels comparable to $Inr_{\lambda 5:1}$ and $Inr_{\lambda 5:2}$ (Figs 2 and 5). However, a third λ5-derived oligonucleotide ($+90_{\lambda 5}$) did not confer any reporter gene activity (Fig. 2). The lack of activity with the latter oligonucleotide may be due to the absence of a transcription start site, lack of c-myc/myn binding and/or non-functional flanking sequences.

The $A_{\lambda 5}$ sequences corresponding to major start sites nos 3 and 4 present in $Inr_{\lambda 5:1}$ (Fig. 1C) show an almost perfect initiator consensus sequence, pyr-$\underline{A}N^T/_A$-pyr (29), except a variation in the last nucleotide ($^{pyr}/_G$). This is also the case for the third major start site no. 5 (Fig. 1B). However, the minor start site no. 7 in $Inr_{\lambda 5:2}$ (Fig. 1C), as well as two other minor start sites (Fig. 1B), do not show an initiator consensus sequence but another sequence $GG\underline{A}^A/_G$. This suggests that, in the context of the whole λ5

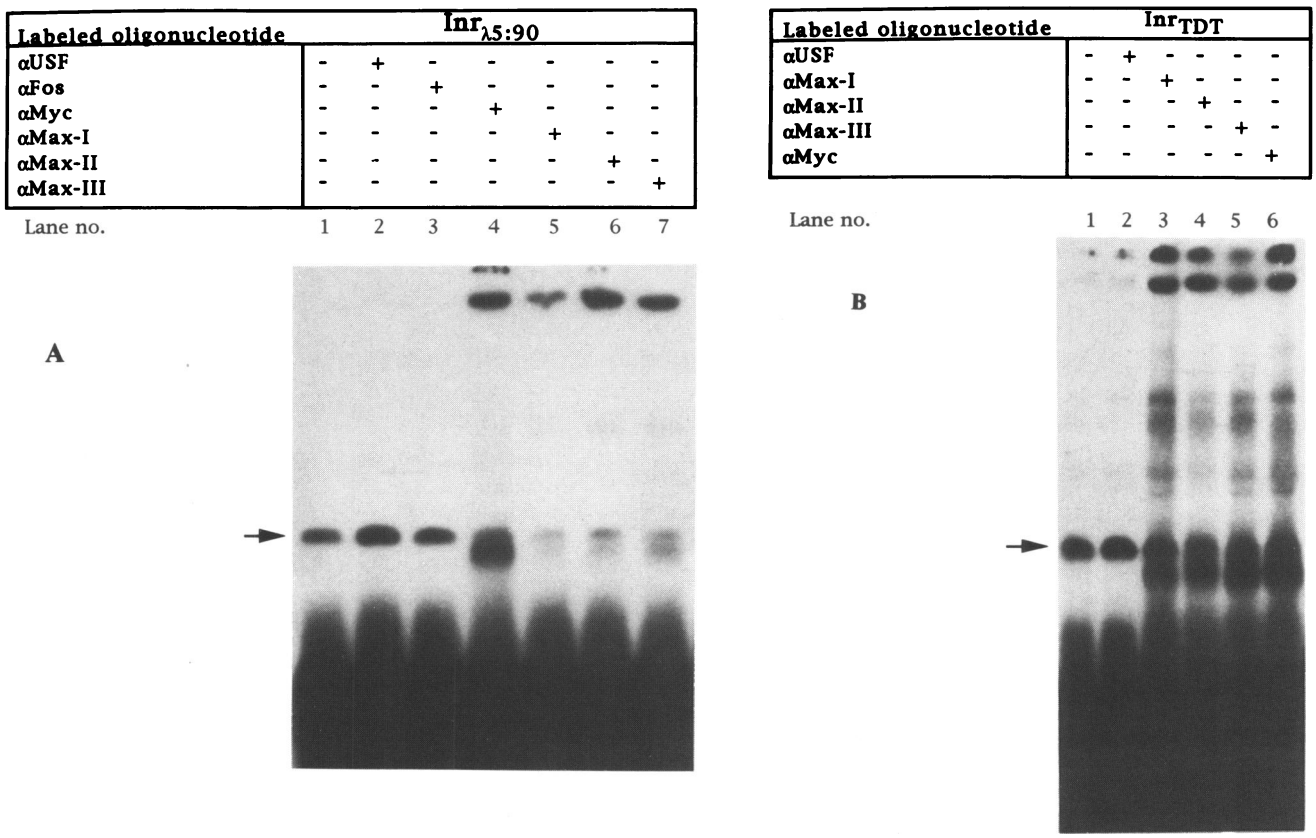


Figure 6. (**A**) Gel retardation analysis of $Inr_{\lambda 5:90}$ in the absence or presence of antibodies. 3 μg nuclear extract from 230-238 pre-B cells were incubated with ^{32}P-end-labelled oligonucleotide $Inr_{\lambda 5:90}$ (lanes 1–7) in either the absence (lane 1) or presence of anti-USF (lane 2), anti-c-Fos (lane 3), polyclonal anti-c-Myc (lane 4) or anti-Max I–III (lanes 5–7, respectively) antibodies (see Materials and Methods). For concentrations of antibodies see Figure 5. Anti-USF was used at 100 ng; polyclonal anti-c-Myc antibodies, 1 μl anti-sera. The B′-like major complex is shown by an arrow. (**B**) Gel retardation analysis of Inr_{TdT} in the absence or presence of antibodies. 3 μg nuclear extract from 230-238 pre-B cells were incubated with ^{32}P-end-labelled oligonucleotide Inr_{TdT} (lanes 1–6) in either the absence (lane 1) or presence of anti-USF (lane 2), anti-Max I–III (lanes 3–5, respectively) or polyclonal anti-c-Myc (lane 6) antibodies (see Materials and Methods). For concentrations of antibodies see (A). The B′-like major complex is shown by an arrow.

promoter the initiator consensus sequences are favoured, and act as major start sites, i.e. nos 3, 4 and 5 (4).

Our experiments show that the initiators $Inr_{\lambda 5:1}$, $Inr_{\lambda 5:2}$ and Inr_{TdT} form several DNA–protein complexes in gel retardation assays. The major complex B′ was found in nuclear extracts from pre-B, B, plasmacytoma and colon carcinoma cells (Figs 3, 4 and 6, and data not shown). This complex was analysed further in extracts from the murine B cell lineage and found to contain c-myc and myn proteins (Figs 4 and 6, and data not shown). Others have reported c-myc binding to the viral AdML initiator in the presence of TFII-I (13).

One candidate binding site for c-myc and myn in $Inr_{\lambda 5:1}$ was the CACCTG E-box motif protected in footprinting analysis (6). Also, CACCTG oligonucleotides shown to bind c-myc–Max heterodimers in DHFR (21) were effective in competition experiments (Fig. 3). Yet, despite mutations of the E-box motif in $Inr_{\lambda 5:1}$, c-myc and myn could still bind. Since (i) footprinting analyses showed protein binding both over the E-box and the transcription start site (6) and (ii) it has been reported that myc, in the presence of TFII-I, can bind to the viral AdML initiator (13), we analysed alternative binding sites. We found, by using modified versions of the $Inr_{\lambda 5:1}$ oligonucleotide, $Inr_{\lambda 5:90}$ and $Inr_{\lambda 5:SV}$, that c-myc/myn proteins could bind to the initiation sites in these oligonucleotides (Fig. 6). In preliminary experiments, using DMS footprints and methylation interference assays, we found with a pre-B cell extract that most of the $Inr_{\lambda 5:1}$ oligonucleotide, except the most 3′ G (or A), was protected (unpublished results). Thus, both transcription initiation sites (nos 3 and 4) and the E-box were protected in $Inr_{\lambda 5:1}$. If all three sites were protected in one and the same DNA molecule is not known. In addition, our analyses of the TdT initiator showed that the transcription start site in both the wild-type and a modified oligonucleotide, Inr_{TdT} and $Inr_{TdT:SV}$, respectively, also bound c-myc/myn proteins (Fig. 6, and data not shown). This finding is supported by preliminary DMS footprint and methylation interference assays with Inr_{TdT}; a pre-B cell nuclear extract protected the transcription start site but not the most 3′ (G)AGA (unpublished results). Furthermore, bacterially produced myc and max fusion proteins bound to the various λ5 and TdT initiators, but not to $+90_{\lambda 5}$ (Fig. 7). This demonstrated that myc and myn/max can bind to the λ5 and TdT initiators and suggested that c-myc and myn belong to the family of IBPs.

All wild-type initiators ($Inr_{\lambda 5:1}$, $Inr_{\lambda 5:2}$ and Inr_{TdT}) promoted expression of the CAT reporter gene. However, the same initiators with modified flanking sequences ($Inr_{\lambda 5:90}$, $Inr_{\lambda 5:SV}$ and $Inr_{TdT:SV}$) did not promote transcription or did so poorly (Fig. 5). Thus, the presence of a transcription start site was not sufficient

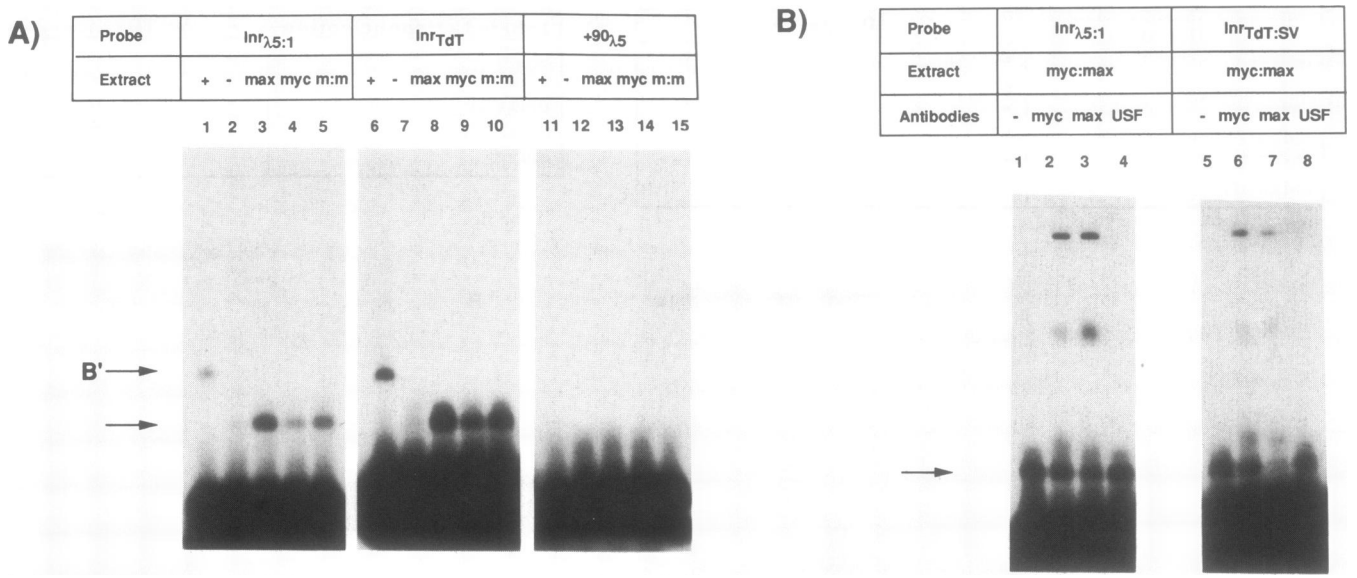


Figure 7. (**A**) Binding of bacterially expressed myc and Max to Inr$_{\lambda 5:1}$ and Inr$_{TdT}$ but not to +90$_{\lambda 5}$. The myc and max expression vectors (fusion proteins) were transformed into bacteria, induced by IPTG and thereafter bacterial extracts were prepared (see Materials and Methods). 1 μg of bacterial extract (see below) was incubated with ^{32}P-end-labelled oligonucleotides Inr$_{\lambda 5:1}$ (lanes 1–5), Inr$_{TdT}$ (lanes 6–10) or +90$_{\lambda 5}$ (lanes 11–15). 2.5 μg nuclear extract from 230-238 pre-B cells was used as control (lanes 1, 6 and 11). The bacterial extracts were prepared from bacteria transformed with fusion vector alone (lanes 2, 7 and 12), max fusion vector (lanes 3, 8 and 13), myc fusion vector (lanes 4, 9 and 14) or a 1:1 mixture of myc and max extracts (lanes 5, 10 and 15). The B′ major complex (nuclear extract) and the complex from bacterially produced myc and max (19), respectively, are shown by arrows. (**B**) Analysis of bacterially expressed myc and max, binding to Inr$_{\lambda 5:1}$ and Inr$_{TdT:SV}$ in the absence or presence of antibodies. The myc and max bacterial extracts in (A) were mixed 1:1 and incubated with ^{32}P-end-labelled oligonucleotides Inr$_{\lambda 5:1}$ (lanes 1–4) or Inr$_{TdT:SV}$ (lanes 5–8) in either the absence (lanes 1 and 5) or presence of polyclonal anti-myc (lanes 2 and 6), polyclonal anti-Max (lanes 3 and 7) or polyclonal anti-USF (lane 4 and 8) antibodies. For concentrations of antibodies see Figures 4 and 6. The B′ major complex (nuclear extract) and the complex from bacterially produced myc/max are shown by arrows.

to promote transcription in transient transfection assays; appropriate flanking sequences were also needed.

The effect of c-myc on the transcriptional activity of the λ5 and TdT initiators was analysed in transient transfections with c-myc expression vectors. Under these conditions, c-myc specifically repressed both the λ5 and the TdT initiators. A mutated c-myc construct lacking the HLH region did not affect reporter gene expression. This is in agreement with *in vitro* transcription assays in which high amounts of bacterially expressed myc were found to repress transcription driven off the AdML initiator (13). In that system myc was dependent on TFII-I for its activity. It is not known if TFII-I is involved in the repression observed in our system. Future investigations will clarify additional proteins involved in modulation of λ5 and TdT transcription initiation both *in vitro* and *in vivo*. The oncoprotein c-myc has been implicated not only in transcriptional repression but also in enhancement of both reporter and cellular genes (this report; 13, 28, 30–36). Thus, it would be interesting to investigate the role of c-myc/myn in the developmental- and cell-type-specific expression of the endogenous λ5 and TdT genes.

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REFERENCES

1 Sakaguchi, N., Berger, C.N. and Melchers, F. (1986) *EMBO J.*, **5**, 2139–2147.
2 Kudo, A., Thalmann, P., Sakaguchi, N., Davidson, W.F., Pierce, J.H., Kearney, J.F., Reth, M., Rolink, A. and Melchers, F. (1992) *Intl. Immunol.*, **4**, 831–840.
3 Kudo, A., Bauer, S. and Melchers, F. (1989) *Prog. Immunol.*, **VII**, 339–347.
4 Mårtensson, I.-L. and Melchers, F. (1994) *Int. Immunol.*, **6**, 863–872.
5 Smale, S.T. and Baltimore, D. (1989) *Cell*, **57**, 103–113.
6 Lo, K., Landau, N.R. and Smale, S.T. (1991) *Mol. Cell. Biol.*, **11**, 5229–5243.
7 Weis, L. and Reinberg, D. (1992) *FASEB J.*, **6**, 3300–3309.
8 Hernandez, N. (1993) *Genes Dev.*, **7**, 1291–1308.
9 Roy, A.L., Meisterernst, M., Pognonec, P. and Roeder, R.G. (1991) *Nature*, **354**, 245–248.
10 Du, H., Roy, A.L. and Roeder, R.G. (1993) *EMBO J.*, **12**, 501–511.
11 Seto, E., Shi, Y. and Shenk, T. (1991) *Nature*, **354**, 241–245.
12 Means, A.L. and Farnham, P.J. (1990) *Mol. Cell. Biol.*, **10**, 653–661.
13 Roy, A.L., Carruthers, C., Gutjahr, T. and Roeder, R.G. (1993) *Nature*, **365**, 359–361.
14 Alt, F., Rosenberg, N., Lewis, S., Thomas, E. and Baltimore, D. (1981) *Cell*, **27**, 381–390.
15 de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. and Subramani, S. (1987) *Mol. Cell. Biol.*, **7**, 725–737.
16 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17 Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA.*, **89**, 5547–5551.
18 Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J.M., Varmus, H. and Lee, W. (1987) *Mol. Cell. Biol.*, **7**, 1697–1709.
19 Blackwood, E.M. and Eisenman, R.N. (1991) *Science*, **251**, 1211–1217.
20 Mårtensson, I.L. and Leanderson, T. (1987) *Eur. J. Immunol.*, **17**, 1499–1502.
21 Mai, S. and Jalava, A. (1994) *Nucleic Acids Res.*, **22**, 2264–2273.
22 Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.

23 Evan, G.J., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) *Mol. Cell. Biol.*, **5**, 3610–3616.
24 Neuberger, M.S. (1983) *EMBO J.*, **2**, 1373–1378.
25 Ephrussi, A., Church, G.M., Tonegawa, S. and Gilbert, W. (1985) *Science.*, **227**, 134–140.
26 Mai, S. (1994) *Gene*, **148**, 253–260.
27 Hariharan, N., Kelley, D.E. and Perry, R.P. (1989) *Genes Dev.*, **3**, 1789–1800.
28 Sigvardsson, M., Johansson, K., Larsson, L.-G., Nilsson, K. and Leanderson, T. (1994) *Leukemia*, **8**, 1157–1163.
29 Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B. and Smale, S.T. (1994) *Mol. Cell. Biol.*, **14**, 116–127.
30 Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I. and Land, H. (1992) *Nature*, **359**, 423–429.
31 Amin, C., Wagner, A.J. and Hay, N. (1993) *Mol. Cell. Biol.*, **13**, 383–390.
32 Benvenisty, N., Leder, A., Kuo, A. and Leder, P. (1992) *Genes Dev.*, **6**, 2513–2523.
33 Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993) *Proc. Natl. Acad. Sci. USA.*, **90**, 7804–7808.
34 Gaubatz, S., Meichle, A. and Eilers, M. (1994) *Mol. Cell. Biol.*, **14**, 3853–3862.
35 Jansen-Dürr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G. and Eilers, M. (1993) *Proc. Natl. Acad. Sci. USA.*, **90**, 3685–3689.
36 Philipp, A., Schneider, A., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Alitalo, K. and Eilers, M. (1994) *Mol. Cell. Biol.*, **14**, 4032–4043.