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In Vitro Transcription of Immunoglobulin Genes in a B-Cell Extract: Effects of Enhancer and Promoter Sequences

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Transfection experiments have led to the identification of three DNA sequences that are responsible for the tissue-specific expression of immunoglobulin genes. As a first step toward characterizing these regulatory phenomena at the biochemical level, we report the development of an in vitro transcription system from cells of the B lymphoid lineage. In these extracts, transcription of the MOPC41 κ promoter is correctly initiated and dependent on the presence of an upstream sequence element located between −44 and −79 base pairs from the cap site. Second, although standard in vitro transcriptions are not affected by the presence or absence of enhancer sequences, we observed that the addition of polyethylene glycol led to a B-cell extract-specific suppression of transcription from a template that carries an immunoglobulin enhancer.

As part of the effort to understand tissue-specific activation and repression of genes, studies on the transcription of DNA transfected into tissue culture cells have begun to define sequences critical for regulating gene expression. For immunoglobulin genes, regulatory sequences have been localized at both the heavy- and the κ light-chain loci. It has been established that the promoters of both the heavy- and light-chain immunoglobulin genes exhibit some tissue specificity (of about 10- to 30-fold) (9, 12, 15, 16, 23). A second element is the tissue-specific enhancer (3, 4, 14, 16, 27, 29, 31–34) located in the intron between the variable and constant regions. Although originally identified as sequences that dramatically increased the levels of transcription of transfected genes, enhancers have now been shown to exhibit other properties as well. For example, several recent reports (19, 43, 44) claim that an enhancer on an endogenous heavy-chain gene may be lost without affecting the transcription level, implying a role for activation but not maintenance of a transcriptionally active state. Further, under certain cellular conditions, the simian virus 40 enhancer (5, 42) as well as the immunoglobulin enhancers (18) have been shown to have repressorlike properties. Finally, a third element has been implicated in the regulation of the heavy-chain gene, but not yet localized (16). Each element appears to be necessary but not sufficient for directing high-level, accurate transcription of a transfected gene. To further understand the roles of these individual elements, a functional in vitro assay would be of great value. Our first goal was, therefore, to derive transcription extracts from a B-lymphoid cell type.

Second, having accomplished that, we were able to examine the role of the immunoglobulin upstream sequence element in the in vitro transcription assay, finding that transcription of a rearranged κ immunoglobulin gene template deleted of its upstream regulatory sequence is decreased about 10-fold relative to the wild-type template. Third, under certain transcription conditions, templates carrying either the heavy-chain or the light-chain enhancer placed upstream from the cap site transcribed less efficiently than those templates deleted of enhancer sequences. This effect was *cis* acting and present only in B-cell extracts derived from EW cells, but not in the heterologous HeLa cell extracts.

Our initial attempts to derive transcription extracts from B cells involved the mouse myeloma MPC 11. We found that neither whole-cell extracts (22) nor nuclear extracts (7) made from this cell line were capable of correctly initiating transcription from a template containing an immunoglobulin promoter. We were able to fractionate away an inhibitor and reconstitute a transcription system, but because the fractionation involved removing potentially important factors from the extract, we did not pursue mouse cells further. The fact that most standard transcription extracts are derived from HeLa cells, a human cell line, prompted us to examine extracts from human B-cell lines for their transcriptional activity. Whole-cell extracts were made from two human Burkitt lymphoma lines, EW and RAMOS, by the procedure of Manley et al. (22). As demonstrated below, these extracts were active.

The templates used for in vitro transcription reactions are diagrammed in Fig. 1A. The template representing the wild-type gene (pK) was derived from the MOPC41 κ gene and contained sequences from approximately 100 base pairs (bp) upstream from the transcription initiation site (endpoint 5′Δ5, Fig. 1A) to the *Bgl*II site in the major $J_κ$-$C_κ$ intron (25). This fragment retains the complete variable region which is rearranged to $J_κ1$, but not the κ enhancer which is further downstream of the *Bgl*II site (4, 33, 34). This short 5′ flank has been shown to be sufficient for accurate initiation and high-level transcription of the gene in a transient transfection assay (4). Deletion analysis of the κ promoter showed previously that important regulatory sequences are present between nucleotides −79 and −44 because deletion 5′Δ7 completely abolished transcriptional competence of the gene while deletion 5′Δ5 had no effect (4). The template representing an inactive promoter mutant (pΔκ) was constructed by engineering a *Hin*dIII site into the 5′ end of 5′Δ7 and cloning the segment of the gene up to the *Bgl*II site into pUC13 cut with *Hin*dIII and *Bam*HI.

To examine transcriptional activity in B-cell extracts, we did runoff transcription reactions at 30°C for 60 min. A

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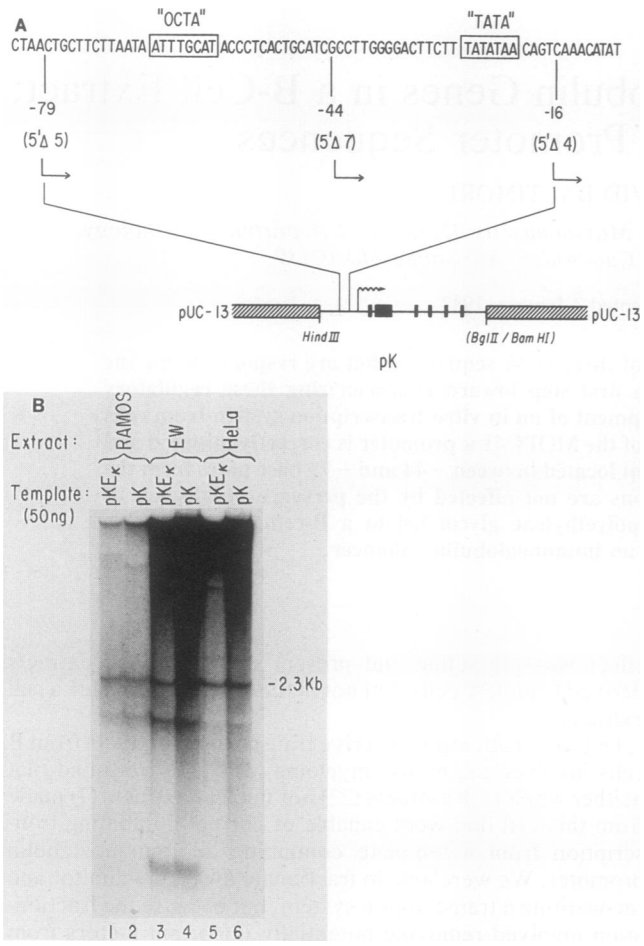

FIG. 1. In vitro transcription in human B-cell extracts. (A) DNA sequence of the promoter region of the MOPC41 κ gene. The deletions 5′Δ5 and 5′Δ7 have been described before (4). The highly conserved octanucleotide sequence which is found upstream of all sequenced heavy- and light-chain variable-region genes is boxed (labeled OCTA). It is located approximately 30 bp upstream from the TATA box. The plasmids pκ and pΔκ were constructed by converting the 5′ ends of 5′Δ5 and 5′Δ7 into a *Hin*dIII site with synthetic linkers followed by cloning the fragment up to the *Bgl*II site in the J_κ-C_κ major intron into *Hin*dIII-*Bam*HI-digested pUC13. pKE_κ and pKE_μ represent plasmids containing either the κ enhancer or the heavy-chain enhancer cloned into the unique *Hin*dIII site of pK. The segments used as the enhancers are an 800-bp *Hin*dIII-*Mbo*II fragment from the J_κ-C_κ intron (25) and a 700-bp *Xba*I-*Eco*RI fragment from the J_H-C_μ intron (3, 14). (B) Transcription in whole-cell extracts made from the human B lymphoma cell lines RAMOS (lanes 1 and 2) and EW (lanes 3 and 4) and from HeLa cells (provided by A. Fire; lanes 5 and 6). The templates were linearized at the *Sac*I site in the polylinker, and the expected 2.3-kb runoff transcript is indicated.

typical reaction mix contained 9 μl (160 μg) of whole-cell extract, 50 μM each ATP, CTP, and GTP, 0.5 μM UTP, 10 μCi of [α-^{32}P]UTP (7,600 Ci/mol), 5 mM creatine phosphate, 0.3 mg of creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.) per ml, 12 mM HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 12% glycerol, 60 mM KCl, 5 mM Mg^{2+}, 1 mM EDTA, 0.6 mM dithiothreitol, linearized template (~50 ng), and poly(dI-dC) (~400 ng). After work up, the RNAs were glyoxalated and analyzed by electrophoresis through a 1.4% agarose gel in 10 mM sodium phosphate (pH 6.8)–1 mM EDTA (22). A runoff transcript of 2.3 kilobases (kb) was evident when RAMOS, EW, or HeLa cell extracts were used (Fig. 1B, lanes 2, 4, and 6). When a κ-chain enhancer sequence was added to the construct, no effect was evident, implying that transcription in these extracts is enhancer independent (Fig. 1B, lanes 1, 3, and 5). (In EW and HeLa cells, the enhancer appears to cause a slight increase in the background radioactivity but not in the 2.3-kb band.) The band at 2.3 kb could be abolished by not adding the template or by transcribing in the presence of 0.5 μg of α-amanitin per ml. Thus it represents a template-specific RNA polymerase II transcript. The band just below 2.3 kb is not decreased by α-amanitin and presumably reflects end labeling of endogenous 18S rRNA.

To assess whether initiation of transcription occurred at the natural cap site, we used a second assay (17). For this assay, the uniformly labeled RNA was hybridized to a single-strand DNA probe spanning the transcription initiation site (generated by cloning the *Pvu*II-*Sau*3A [33] fragment of the κ gene into bacteriophage M13). The resulting complex was digested with RNase T_1, and the RNase-

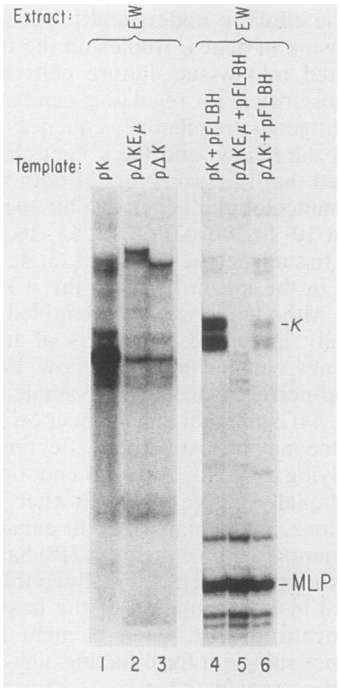

FIG. 2. Effect of the upstream deletion 5′Δ7 on in vitro transcription in B-cell extracts utilizing a preincubation pulse-chase protocol. Runoff transcripts were obtained with templates containing either the wild-type promoter (lane 1) or the truncated κ promoter (lanes 2 and 3). Lanes 4 to 6, In vitro transcription with closed circular templates containing the wild-type promoter (lane 4) or the truncated κ promoter (lanes 5 and 6). In these reactions 50 ng of a closed circular template containing the adenovirus major late promoter (MLP) was included as an internal control. The transcripts specific to the κ template or the adenovirus template are indicated as κ and MLP, respectively. For a template containing the major late promoter we used the plasmid pFLBH which contains sequences from 14.7 to 17.0 map units of adenovirus inserted between the *Bam*HI and *Hin*dIII sites of pBR322 (kind gift of A. Fire and M. Samuels). The upper of the two bands (κ) derived from the immunoglobulin promoter represents the correct start for κ transcription. The lower band is seen at variable intensities and probably does not represent a different cap site, as explained in the text.

resistant RNA fragments were analyzed by electrophoresis through a 6% polyacrylamide gel with 8.3 M urea. Analysis of in vitro-synthesized RNA by this method is shown in Fig. 2 (lane 4). The upper band (labeled κ) represents the correct cap site. The band just below it was seen at variable intensities (e.g., see Fig. 3D) and probably does not represent a different cap site but rather arises from cleavage with RNase T_1 at the next G residue from the 3′ end of the protected region. (Examination of the sequence near the *Sau*3A1 site [33] shows that the second set of G residues on the RNA lies 19 bp upstream from the end of the region of homology with the single-stranded DNA probe.) Thus, the extracts generated from B cells were capable of correctly initiating and transcribing the immunoglobulin promoter in vitro with approximately the same efficiency as a HeLa cell extract.

To analyze the effect of 5′-flanking sequences in vitro, we examined the transcription of the deleted gene, pΔκ. Because many regulatory effects act on the rate of initiation of transcription, we chose to use a preincubation, pulse-chase protocol which measures initiation rates (11). Either the linearized or the supercoiled template (50 ng) was incubated in a volume of 20 μl with 9 μl (~160 μg) of EW extract, 6% (wt/vol) polyethylene glycol (PEG) 20,000, and all other components described above except the nucleotides for 60 min at 30°C. Transcription was then initiated by the addition of nucleotides and radiolabeled UTP (final concentration, 60 μM each ATP, CTP, and GTP, 1 μM UTP, and 10 μCi of [α-^{32}P]UTP). The initiating pulse was maintained for 10 min at 30°C followed by a 10-min chase with a vast excess of nonradioactive nucleotides (final concentrations, 330 μM ATP, CTP, and GTP, 1 mM UTP). The reactions were quenched, and the transcripts were analyzed by electrophoretic separation. Incorporated radioactivity in this assay is proportional to the number of correct initiations occurring during the pulse. In Fig. 2, comparison of lanes 2 and 3 with lane 1 shows that the template pκ, which contains about 100 bp upstream of the initiation site, initiated approximately 10-fold more efficiently than did the deletion mutant pΔκ. Again, the presence of the heavy-chain enhancer placed at −44 bp to the truncated promoter did not alter the level of transcription. (The upper band present in all lanes presumably arises from end-to-end transcription of the linearized plasmid which is often observed in such reactions and is independent of the presence of regulatory sequences). When closed circular templates were used, a similar effect of the promoter truncation was observed (Fig. 2, lanes 4 to 6). In these reactions a template containing the major late promoter of adenovirus was included as an internal control (28). Major late promoter-specific transcripts were detected with the M13 clone XH11 (provided to us by A. Fire and M. Samuels), and the expected protected RNA fragment of 180 bp is labeled MLP. Comparison of lanes 5 and 6 with lane 4 shows that there was a 10-fold decrease in the efficiency of transcription from the mutant promoter, whereas the transcript of the major late promoter remained constant. The reason for the apparent decrease in the amount of transcription from the supercoiled template containing the heavy-chain enhancer was not further addressed. It is evident, however, that the dependence of transcription on an upstream sequence between −44 and −79 is observed whether the template is linear or circular. To assess whether the effect described above was specific to B-cell extracts, we transcribed the same templates in the heterologous HeLa whole-cell extract. A four- to fivefold decrease in transcription was seen with the deleted template when compared with the wild-type template (data not shown). Thus, the effect of the deletion is, at best, only modestly tissue specific.

In EW cell extracts, the same level of RNA was generated from two templates, one carrying and one deleted of the immunoglobulin κ enhancer (Fig. 1B). This was true in HeLa extracts as well and was independent of whether the template was linear or closed circular (data not shown). In attempts to show enhancer effects in vitro, we varied a number of obvious conditions individually, e.g., Mg^{2+}, Ca^{2+}, NH_4^+, spermidine, time, but none of these conveyed any template selectivity. However, as described below, the addition of PEG to the transcription reaction did lead to a suppression of transcription from a template carrying either the heavy- or light-chain enhancer.

The plasmid pBRK was constructed by cloning the 2-kb *Pvu*II-*Bgl*II fragment of the rearranged MOPC41 κ gene containing the promoter, variable region, and extra J_κ segments in pBR322. The κ enhancer, present on an 800-bp *Hin*dIII-*Mbo*II fragment of the J_κ-C_κ intron (25), was inserted into the unique *Hin*dIII site in pBRK to generate the plasmid pBRKE_κ (Fig. 3A). The orientation of the insert was determined by cleaving with *Ava*II which cuts once within the enhancer and placed the essential 250 bp (marked by the cross-hatched box) approximate 600 bp away from the cap site. The plasmid pKE_μ (Fig. 3B) was constructed from plasmid pK by adding the 700-bp *Xba*I-*Eco*RI heavy-chain complete enhancer fragment from the J_μ-C_μ intron (16) upstream of the start site of transcription at the unique *Hin*dIII site. The orientation of this insert placed the essential 300 bp (marked by the cross-hatched box) that retains 30 to 50% of the enhancer activity approximately 130 bp away from the cap site.

To probe the effects of the enhancer sequences on the initiation step of the transcription reaction, we again followed the preincubation pulse-chase protocol. When the two sets of plasmids described above were used as templates for in vitro transcription reactions in the presence of 6% PEG 20,000, a striking difference in the extent of RNA produced from each template was observed (Fig. 3C). The presence of either the κ enhancer (lanes 1 and 2) or the heavy-chain enhancer (lanes 3 and 4) on a template led to a suppression of transcription from that template. Most interestingly, this effect was limited to transcription in a B-cell extract because when the same templates were transcribed in a HeLa cell extract, no difference in the levels of RNA was observed (lanes 5 and 6). Use of closed circular plasmids as DNA templates yielded the same results. In a B-cell extract, the plasmid containing the heavy-chain enhancer transcribed 5- to 10-fold less efficiently (Fig. 3D, lane 1) than a plasmid lacking an enhancer (lane 2). Use of a plasmid (pFLBH) containing the adenovirus major late promoter as an internal control showed that the extent of transcription of this promoter (lanes 1 and 2, labeled MLP) remained constant. The repressive effect of the immunoglobulin enhancer was observed only in the B-cell extract and not in HeLa extracts (Fig. 3D, lanes 3 and 4). This effect was reproducibly observed if the transcription conditions were kept constant with a given cell extract. To observe maximal suppression with an independently derived extract from the same cell line, we often had to adjust the ratios of DNA template and protein in the extract. This negative enhancer effect in B-cell extracts was observed in the range of 50 to 150 mM KCl, but was lost if the protein concentration in the reaction was decreased by a factor of 4 from its optimal concentration. Although transcription reactions in HeLa cell extracts were done under a wide variety of different conditions, we never

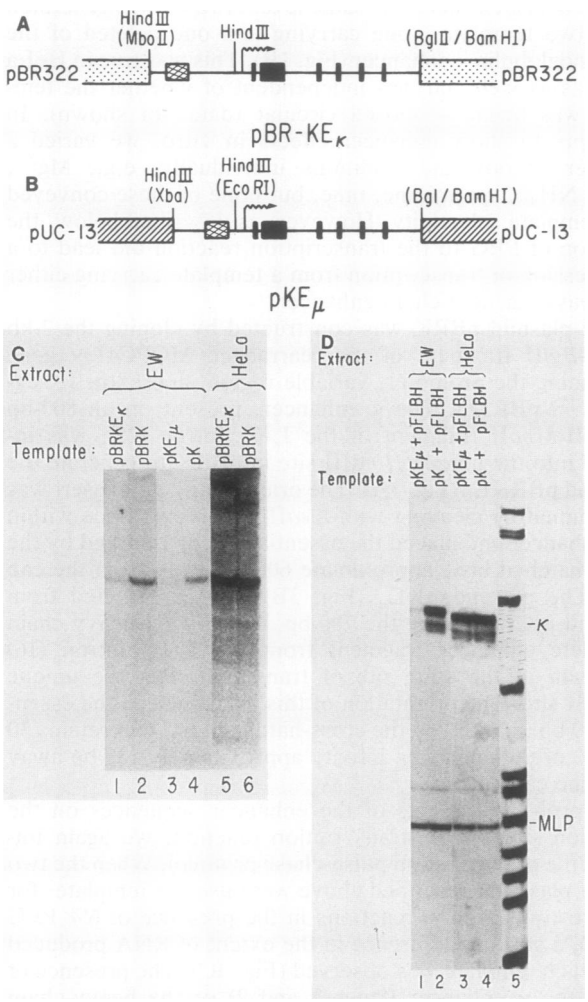

FIG. 3. Plasmids utilized for in vitro transcription reactions and effect of immunoglobulin enhancer sequences in vitro. (A) pBRKE$_\kappa$ is a plasmid carrying the κ enhancer which was derived from pBRK as described in the text. The cross-hatched box represents the essential 250 bp of the κ enhancer. pBRK and pBRKE$_\kappa$ were linearized for runoff transcription assays by cutting with *Sal*I in the pBR322 backbone. (B) pKE$_\mu$ is a μ enhancer-bearing plasmid and was derived from pκ (Fig. 1A). The cross-hatched box represents the essential 300 bp of the μ enhancer. Plasmids pK and pKE$_\mu$ were linearized for runoff transcription assays by cutting with *Sac*I in the polylinker. (C) Effect of immunoglobulin enhancer sequences on in vitro transcription with linear templates. Runoff transcripts were obtained with pBRKE$_\kappa$ and pBRK as templates in B-cell extracts (lanes 1 and 2) or in a HeLa cell extract (lanes 5 and 6) or with pKE$_\mu$ and pK as templates in a B-cell extract (lanes 3 and 4). (D) Effect of immunoglobulin enhancer sequences on in vitro transcription with closed circular templates and the preincubation pulse-chase protocol. κ-gene-specific radioactive RNA was selected by hybridization to a single-stranded DNA probe spanning the initiation site of transcription. A plasmid containing the adenovirus major late promoter was included as an internal control in all transcription reactions, and the resultant RNA is labeled MLP. RNA (labeled κ) was synthesized from templates with and without the immunoglobulin heavy-chain enhancer in a B-cell extract (lanes 1 and 2) and in a HeLa extract (lanes 3 and 4). Lane 5, End-labeled, *Hpa*II-digested pBR322 DNA.

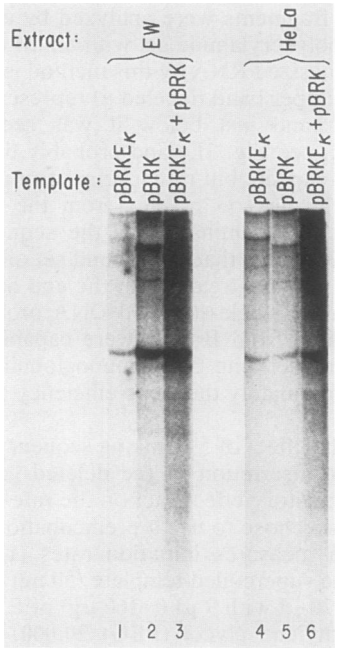

FIG. 4. Runoff transcription analysis of the effect of mixing templates with and without the κ enhancer sequence. Approximately 50 ng each of linearized templates pBRKE$_\kappa$ and pBRK were transcribed individually (lanes 1 and 2, respectively) or as an equimolar mixture (lane 3) in a B-cell extract or in a HeLa cell extract (lanes 4, 5, and 6). Transcriptions were done with 45% (vol/vol) whole-cell extract and 25 μg of poly(dI-dC) · poly(dI-dC) per ml in the presence of 6% (wt/vol) PEG 20,000 following the preincubation, pulse-chase protocol. The in vitro-synthesized RNA was glyoxalated and analyzed by electrophoresis through a 1.4% agarose gel.

detected differential transcription of templates as a consequence of the presence of an immunoglobulin enhancer.

To examine whether the suppression of transcription was mediated in *cis* upon the template carrying the enhancer, we did experiments mixing templates pBRK and pBRKE$_\kappa$ in the same reaction. If the suppression described earlier was due to depletion of some limiting transcription factor by binding to the enhancer sequences, then a second template in the reaction should also be adversely affected (i.e., in *trans*). However, this was not so (Fig. 4). A negative effect of the presence of the enhancer on the transcription of linearized templates pBRK and pBRKE$_\kappa$ in individual reactions was observed (Fig. 4, lanes 1 and 2). However, when both templates were present simultaneously in the same transcription reaction, the amount of RNA observed was similar to that observed with pBRK alone (compare lanes 3 and 2), thus showing that transcription of this template was not repressed owing to its presence in the same reaction as pBRKE$_\kappa$. When the same experiment was done in a HeLa extract, once again the results were additive (Fig. 4, lanes 4, 5, and 6). In this case there was no pBRKE$_\kappa$ template-specific repression, thus yielding runoff transcripts of equivalent amounts with either pBRK or pBRKE$_\kappa$ (lanes 4 and 5) and approximately double the amount in the mixed transcription reaction (pBRK and PBRKE$_\kappa$) (lane 6). Furthermore, the fact that the levels of transcripts initiating from the major late promoter (Fig. 3D) were unchanged in the presence of both plasmids pBRK and pBRKE$_\kappa$ also argues against the negative effect being mediated in *trans* by the depletion of some limiting transcription factor. We therefore

suggest that the observed repressive effect is *cis* acting upon the plasmid that carries an immunoglobulin enhancer sequence and is specific to an extract derived from a B lymphoid cell.

We reported here the development of transcriptionally competent whole-cell and nuclear extracts from two independent human B-cell lymphomas which should be useful for the biochemical analysis of tissue-specific regulatory phenomena. In such extracts, transcription from the promoter of the MOPC41 κ gene was correctly initiated, and a promoter deletion significantly reduced the level of initiated RNA. Furthermore, in the presence of 6% PEG, templates carrying either the μ or κ enhancer transcribed less efficiently than those deleted of enhancer sequences. This suppressive effect was restricted to B-cell extracts only and not observed in heterologous HeLa cell extracts. These effects may now be used as functional assays during purification of the putative factors that mediate them and thus set the stage for a more refined biochemical and mechanistic analysis of immunoglobulin gene transcription.

The sequences responsible for the tissue selectivity of the immunoglobulin promoters have been narrowed down to a 150-bp fragment for the V_H promoter (16) and a 220-bp fragment for the V_κ promoters (12, 15). A strikingly conserved octanucleotide sequence (ATTTGCAT) motif appears in these fragments and has been postulated to be a recognition sequence for a tissue-specific regulatory protein (10, 30). It is curious, therefore, that deletion of this sequence causes similar effects on the amounts of transcripts generated in vitro in either B-cell extracts or the heterologous HeLa extract. This observation, however, is consistent with the observation that both EW cells and HeLa cell nuclear extracts contain a factor that binds specifically over this sequence (41). Further, the same octamer sequence motif has been shown to be of importance for the regulation of non-B-cell-specific genes as well, e.g., the U1 and U2 small nuclear RNA genes (1, 24). Therefore, it is not surprising that it binds a non-tissue-specific factor.

Hydrophilic polymers such as PEG and polyvinyl alcohol have been essential components of in vitro replication reactions in crude extracts (13) and more recently have been used to increase the efficiency of in vitro splicing reactions (20). These polymers are believed to have two effects on these reactions. First, they promote the concatenation of DNA into large aggregates (21) induced presumably by initial condensation, and second, they increase the local concentration of all macromolecules by an excluded volume effect that has been called macromolecular crowding (45). What the role of PEG is in bringing about an enhancer-dependent suppression of in vitro transcription is not clear. It is conceivable that compaction may make it easier for distant sections of the DNA to interact with each other, and macromolecular crowding may help bring about the binding of relatively rare proteins with these sequences. Recently, the simian virus 40 enhancer (35–37, 40) and the immunoglobulin μ enhancer (2, 37) have been shown to have a positive effect on in vitro transcriptions. Examinations of the conditions required to observe an enhancement in vitro shows (i) the need to concentrate the nuclear extract and (ii) a strong dependence on the presence of spermidine. Since the latter is believed to promote condensation of DNA, the combination should lead to conditions rather similar to those generated in our reactions by the addition of PEG.

The paradoxical nature of extract-specific suppression of in vitro transcription from a template carrying an immunoglobulin enhancer requires some speculative explanation. Even in experiments with the simian virus 40 enhancer only a subset of the functional characteristics of that enhancer have been mimicked in vitro. This has led to the argument that perhaps an enhancer can operate at various levels (39), and at the present state of refinement of in vitro systems only a few of these can be mimicked simultaneously. More specifically, there is evidence to suggest that functional activation of an enhancer is a multicomponent phenomenon in which there is a need for tissue-specific as well as nonspecific *trans*-acting factors (26, 38). One possibility is that we are reconstituting only partial function under our assay conditions by only a subset of these factors interacting with the enhancer, thus giving rise to the unexpected observed inhibition. Further, it has recently been shown that both the simian virus 40 and immunoglobulin enhancers are the sites of negative regulation of transcription under certain cellular conditions (5, 18, 42). Perhaps our in vitro conditions mimic more closely these inhibitory conditions rather than those that give enhancement. Another possibility is that binding of the correct set of factors is inducing an inappropriate function because our assay is biased by our preconceived notion of what the function of an enhancer is. For example, if the role of the protein bound to the μ enhancer (6, 8) is not to serve as an entry site for RNA polymerase but rather to define nuclear localization or to change chromatin structure, then its effect in vitro would not necessarily be seen as an increase in the number of polymerase molecules accurately initiating transcription during the pulse in our experiments.

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