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The Ubiquitous Octamer-Binding Protein(s) Is Sufficient for Transcription of Immunoglobulin Genes

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All immunoglobulin genes contain a conserved octanucleotide promoter element, ATGCAAAT, which has been shown to be required for their normal B-cell-specific transcription. Proteins that bind this octamer have been purified, and cDNAs encoding octamer-binding proteins have been cloned. Some of these proteins (referred to as OTF-2) are lymphoid specific, whereas at least one other, and possibly more (referred to as OTF-1), is found ubiquitously in all cell types. The exact role of these different proteins in directing the tissue-specific expression of immunoglobulin genes is unclear. We have identified two human pre-B-cell lines that contain extremely low levels of OTF-2 yet still express high levels of steady-state immunoglobulin heavy-chain mRNA in vivo and efficiently transcribe an immunoglobulin gene in vitro. Addition of a highly enriched preparation of OTF-1 made from one of these pre-B cells or from HeLa cells specifically stimulated in vitro transcription of an immunoglobulin gene. Furthermore, OFT-1 appeared to have approximately the same transactivation ability as OTF-2 when normalized for binding activity. These results suggest that OTF-1, without OTF-2, is sufficient for transcription of immunoglobulin genes and that OTF-2 alone is not responsible for the B-cell-specific regulation of immunoglobulin gene expression.

The octamer motif, ATGCAAAT (8, 33), has been found in a variety of eucaryotic regulatory elements, including promoters, enhancers, and origins of replication. Some of these elements are constitutively active in all cell types, whereas others direct cell cycle-regulated or tissue-specific activity. In the case of the small nuclear RNA genes and the histone H2b gene, deletion of the octamer greatly reduces transcription and, in the latter case, also eliminates cell cycle regulation (1, 10, 22, 42). In both the heavy- and light-chain immunoglobulin gene promoters and in the heavy-chain enhancer, an intact octamer hs been shown to be necessary to direct B-cell-specific transcription (8, 12). Several proteins that specifically bind the octamer sequence have been identified in nuclear extracts from mammalian cells. One such protein, termed octamer transcription factor (OTF-1; also called oct-1, NFA-1, OBP100, and NF III), is found in all tissue types tested; at least one other, termed OTF-2 (also called oct-2 and NFA-2), is found almost exclusively in lymphoid cells (23, 32, 37, 45, 46).

Recently, several cDNAs encoding these octamer-binding proteins have been isolated from both B-cell lines and a teratocarcinoma cell line (4, 29, 39, 47). Sequence analyses of these cDNAs reveal that these genes belong to an even larger group of DNA-binding proteins called the POU family. The POU family of proteins also includes the Pit-1 protein, which is expressed in pituitary cells and is involved in the transactivation of growth hormone and prolactin genes, and the *unc-86* protein, which is a nematode gene product involved in neuronal development (2, 9, 16). All of these genes contain a conserved homeobox domain, shown to be important in DNA binding through its helix-turn-helix motif, and a POU-specific domain of unknown function but which has been implicated in contributing to DNA-binding activity (21, 48). In addition to their structural relatedness, the four identified POU proteins also seem to be related functionally. At least three (OTF-1, OTF-2, and Pit-1) are transcriptional transactivators and bind to similar sequences, whereas OTF-2, Pit-1, and the *unc-86* protein have been shown to direct tissue-specific activities (for reviews, see reference 14).

The fact that OTF-2 is found only in lymphoid cells and that the octamer is conserved in all immunoglobulin promoters examined has led to the hypothesis that this protein mediates the tissue specificity of immunoglobulin gene expression. Recent evidence, however, suggests that OTF-1 can transactivate immunoglobulin gene promoters in vitro (25). In that report, crude preparations of octamer-binding proteins were made from HeLa cells, which contain almost exclusively OTF-1, and from BJA-B cells, a B-cell line containing high levels of OTF-2. When either of these preparations was added to HeLa or B-cell nuclear extracts depleted of octamer-binding proteins, stimulation of immunoglobulin gene transcription in vitro occurred. These results differ from other reports suggesting that only OTF-2, and not OTF-1, functions to stimulate immunoglobulin gene transcription (39, 40).

To resolve these conflicts and further examine the role of the different OTFs in immunoglobulin gene expression, an in vitro transcription system was developed. Using nuclear extracts from cell lines containing varied ratios of OTF-1 to OTF-2, we show that the level of OTF-2 in lymphoid cells does not correspond to the ability of the cells to transcribe an immunoglobulin gene in vitro. Furthermore, we show that a highly purified preparation of OTF-1 can specifically transactivate an immunoglobulin gene when added to lymphoid nuclear extracts but does not stimulate transcription from a non-octamer-containing promoter. When this OTF-1 preparation or a partially purified nuclear fraction containing only binding activity associated with OTF-2 is added to HeLa nuclear extract, very little stimulation of immunoglobulin transcription can be detected. These results indicate that although octamer-binding proteins are required for immuno-

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globulin gene transcription, they are not sufficient for their tissue-specific expression.

MATERIALS AND METHODS

Cell lines. SMS-SB is a clonally derived human pre-B-cell line from a patient with acute lymphoblastic leukemia (43). Nalm-6 is also a human acute lymphoblastic leukemia pre-B-cell line (15). Both lines are prototypically pre-B in that they contain no light-chain immunoglobulin gene rearrangements (P. Fell and P. Tucker, unpublished results). Namalwa is an African Burkitt's lymphoma cell line; HeLa is a human cervical carcinoma cell line. BCL_1 is a spontaneously derived murine B-cell leukemia (20). Namalwa and Nalm-6 were propagated in vitro in RPMI containing 10% fetal calf serum and antibiotics. SMS-SB cells were grown in serum-free Iscones modification of minimal essential medium supplemented with insulin (10 μg/ml), transferrin (30 μg/ml), bovine serum albumin (10 μg/ml), and ethanolamine (20 mM) (52).

Plasmids, probes, and oligonucleotides. The template plasmid for the in vitro transcription assay was made by cloning the 593-base-pair (bp) *Bam*HI-*Xba*I fragment of the immunoglobulin heavy-chain gene (20) expressed in the BCL_1 cell line into pUC19. This fragment contains 270 bp upstream of the transcription initiation site, including the conserved heptamer and octamer sequences. In the opposite orientation, a 420-bp *Bam*HI-*Nru*I fragment of the Rous sarcoma virus (RSV) long terminal repeat (LTR) from pRSVCAT was inserted to make $pBCL_1RSV$. Antisense RNA probes were made by cloning DNA fragments into the pGEM4 vector (Promega Biotec, Madison, Wis.). For the BCL_1 V_H probe, a 322-bp *Bam*HI-*Nco*I fragment containing 55 bp downstream of the major transcription initiation site was used, generating $pBCL_1$-5′. This plasmid was linearized with *Hin*fI and transcribed with SP6 polymerase (Promega) to make a 95-nucleotide-long labeled RNA probe. For the RSV probe, the entire 420-bp RSV LTR fragment was used, generating pGEMRSV. This plasmid was linearized with *Eco*RI and transcribed with T7 RNA polymerase to make a 113-nucleotide-long labeled RNA probe. Oligonucleotides were made on an Applied Biosystems 380A oligonucleotide synthesizer. The double-stranded octamer oligonucleotide contained the sequence ATGAATATGCAAACAGGTG and its complementary strand. The mutated oligonucleotide contained the sequence GAATCACAGCAAATCATA and its complementary strand.

Gel migration inhibition assay. The gel migration inhibition assay was performed as previously described (13, 23), using a 3′-end-labeled 322-bp *Bam*HI-*Nco*I restriction fragment. This fragment was derived from plasmid PMK3 (13, 23), which contains the heavy-chain variable-region gene expressed in the BCL_1 cell line.

Northern (RNA) blot analysis. The human Cμ probe used in Northern blot analysis was a 1-kilobase-pair (kb) *Xba*I fragment containing portions of the Cμ2 and Cμ3 domains (50). The oct-2 probe was a gift from L. Staudt and consists of a 3.1-kb cDNA clone (21). The β-actin probe was a 700-bp *Pvu*II fragment from a human cDNA clone. RNA was isolated by the guanidinium-hot phenol method (27), poly $(A)^+$ selected on $poly(dT)_{12-18}$ (Pharmacia, Inc., Piscataway, N.J.), and separated on a 1% agarose–6% formaldehyde gel. The RNA was transferred to GeneScreen (Dupont, NEN Research Products, Boston, Mass.), and the filter was prehybridized at 42°C for 4 h in 50% formaldehyde–1% sodium dodecyl sulfate (SDS)–1 M sodium chloride–10% dextran. The labeled probe was added, and hybridization was continued for 24 h at 42°C. Hybridized filters were washed twice in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, once in 2× SSC–1% SDS at 60°C for 60 min, and twice in 0.01% SSC. After autoradiography, filters were stripped by boiling in water for 10 min.

Purification of octamer-binding proteins from nuclear extracts of SMS-SB and HeLa. The nuclear extracts were prepared as previously described (5). Affinity-purified octamer-binding proteins from SMS-SB and HeLa cells were prepared as described previously (13), with modifications. Briefly, approximately 80 mg of nuclear protein derived from each cell line was passed over a 8-ml DEAE column equilibrated with buffer D. The octamer-binding activity flowed through the column under these conditions and was subsequently applied to a 4-ml column containing heparin-agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The column was washed, and the bound octamer-binding proteins were eluted with 2 column volumes of buffer D containing 0.4 M KCl. The eluted protein was precipitated with 50% ammonium sulfate, the solubilized pellet was applied to a 300-by-7.5-mm Bio-Sil TSK 250 column, and fractions containing octamer-binding activity were pooled and applied to a sequence-specific DNA affinity column prepared by using a 65-bp 5′-aminoethyl oligonucleotide attached to cyanogen-activated Sephacryl CL-2B-300. DNA-affinity chromatography was performed essentially as described previously (3), with modifications. Briefly, the pooled TSK-250 fractions containing octamer-binding activity were equilibrated to 0.25 M KCl in buffer D, and heparin sulfate was added to a concentration of 0.4 mg/ml as a competitor for nonspecific DNA-binding proteins. The mixture was allowed to stand for 10 min at 4°C and then was added to a 3-ml DNA-affinity column. The bound proteins were washed with at least 5 column volumes of buffer D containing 0.25 M KCl, and the octamer-binding activity was eluted by sequential additions of buffer D containing 1 and 0.5 M KCl. The 1 and 0.5 M eluates were pooled and equilibrated to 0.1 M KCl for a second pass over a 1-ml affinity column in the presence of 0.2 mg of heparin per ml.

In vitro transcription. In vitro transcription reactions were performed essentially by the method of Dignam et al. (5), with some modifications. After isolation of crude nuclei, the pellets were suspended in buffer C at a 1:1 volume ratio to obtain nuclear extracts with a final concentration of approximately 10 mg/ml. Nuclear extracts were dialyzed twice in buffer D for at least 2 h, frozen in liquid nitrogen, and stored at −70°C. Reaction mixtures contained 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 12 mM $MgCl_2$, 600 μM ATP, UTP, GTP, and CTP, 40 U of RNasin (Promega), 1 μg of uncut template DNA, and approximately 300 μg of nuclear protein in a final volume of 50 μl. The reaction mixtures were incubated for 1 h at 30°C. The protein was extracted twice with phenol-chloroform (1:1), and the nucleic acids were ethanol precipitated, washed with 70% ethanol, and suspended in 26 μl of water. The DNA was digested by addition of 3 μl of 10× DNase buffer (400 mM Tris hydrochloride [pH 7.9], 100 mM NaCl, 60 mM $MgCl_2$) and 1 U of RQ1 DNase (Promega) and incubation for 15 min at 37°C. After one phenol-chloroform extraction, the RNA was ethanol precipitated.

RNase protection assay. In vitro-generated RNA was suspended in 30 μl of hybridization buffer containing 80%

formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.7), 400 mM NaCl, 1 mM EDTA, and approximately 10^6 cpm of antisense RNA probe. The RNA mixture was heated to 90°C for 2 min and placed directly at 60°C overnight. After hybridization, 300 μl of digestion buffer containing 10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 300 mM NaCl, 40 μg of RNase A (Sigma Chemical Co., St. Louis, Mo.) per ml, and 2,500 U of RNase T_1 (Bethesda Research Laboratories) was added, and digestion was continued for 1 h at 37°C; 50 μg of proteinase K (Sigma) and SDS (0.6%, final concentration) was added, and the preparation was incubated for 15 min at 37°C. After phenol-chloroform extraction, RNA was ethanol precipitated and suspended in 80% formamide and dyes. Protected RNA fragments were separated on a 6% acrylamide gel containing 50% urea, and autoradiography was performed for 12 to 48 h.

RESULTS

Some pre-B-cell lines lack OTF-2. Through gel retardation assays using nuclear extracts made from a variety of lymphoid cell lines, we have identified two pre-B-cell lines, SMS-SB and Nalm-6, that express very low levels of OTF-2 relative to a phenotypically more mature Burkitt's lymphoma cell line, Namalwa (Fig. 1A). The probe used in this assay is an end-labeled fragment of the BCL_1 immunoglobulin heavy-chain gene containing the conserved octamer and heptamer sequences. Species 1 (S1) corresponds to a protein dimer complex formed by the cooperative binding of OTFs to both the octamer and the heptamer, whereas S2 and S3 correspond to monomeric OTF-1 and OTF-2, respectively (18, 24, 34, 35). Addition of a oligonucleotide containing the octamer sequence, but not one containing a mutated octamer, specifically inhibited the formation of all three species (Fig. 1A, lanes 3, 4, 6, 7, 9, 10, 12, and 13). A nonspecific band (NS in Fig. 1A) that ran closely with S3 was seen in some experiments, but this band was not abolished by the octamer-containing oligonucleotide. The underloading of Nalm-6 nuclear protein in this experiment did not correlate with the level of OTF-1 in this cell line, which was approximately the same as the levels in Namalwa, SMS-SB, and HeLa cells. The level of OTF-2-binding activity in these pre-B-cell lines was comparable to the low level detectable in some nonlymphoid cell lines such as HeLa, yet all three cell lines (SMS-SB, Nalm-6, and Namalwa) accumulated similar amounts of steady-state immunoglobulin heavy-chain mRNA in vivo, as determined by Northern blot analysis when normalized to β-actin message (Fig. 1B; compare lanes 1, 2, and 3). Reprobing the blot with an OTF-2-specific probe confirmed that the level of OTF-2 mRNA corresponded to the amount of OTF-2-specific binding activity in each cell line (Fig. 1B). Even though Namalwa was underloaded and SMS-SB was overloaded (when normalized to β-actin levels in Nalm-6 and HeLa cells), the OTF-2-specific, 7-kb message was found only in the Namalwa lane. This finding is in agreement with others who detected this message only in cell lines containing OTF-2-binding activity (29, 39, 44).

In vitro transcription of an immunoglobulin gene does not correspond to OTF-2 expression. The plasmid used as a template in the in vitro transcription assay (Fig. 2) contains the expressed V_H promoter from the BCL_1 cell line. As an internal control, the RSV LTR was inserted into the template plasmid in the opposite orientation. The RSV LTR does not contain a consensus octamer motif and has been shown to bind the general transcription factor C/EBP (38) and to be active in both lymphoid and nonlymphoid cells.

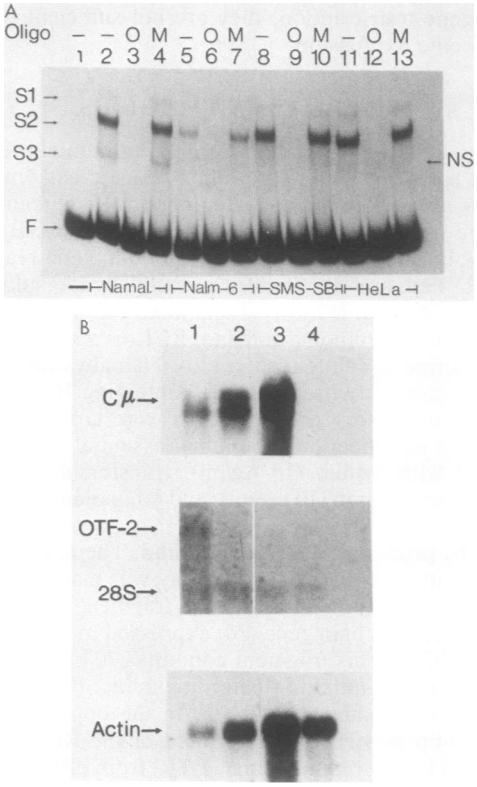

FIG. 1. Gel retardation and Northern blot analysis of OTF-2. (A) An end-labeled 322-bp fragment from the BCL_1 heavy-chain promoter was incubated with no nuclear extract (lane 1) or with Namalwa (lanes 2 to 4), Nalm-6 (lanes 5 to 7), SMS-SB (lanes 8 to 10), or HeLa (lanes 10 to 13) nuclear extract. Competitor oligonucleotides containing the octamer motif (O; lanes 3, 6, 9, and 12) or a mutated octamer motif (M; lanes 4, 7, 10, and 13) were added to demonstrate the specificity of the three species for the octamer. (B) Poly(A)$^+$ RNA was electrophoresed on a 1% agarose-formaldehyde gel and transferred to GeneScreen (Dupont, NEN). The same blot was hybridized with a μ constant-region probe (Cμ), stripped and hybridized with an OTF-2 probe, stripped again, and hybridized to a β-actin probe. Lanes: 1, Namalwa; 2, Nalm-6; 3, SMS-SB; 4, HeLa. NS, Nonspecific band.

The antisense RNA probes used to detect the in vitro-generated transcripts span the transcription initiation start sites of both genes and are shown, with their sizes in nucleotides, below the template plasmid (Fig. 2). Also shown are the sizes of two of the major protected fragments that were formed after the RNase protection assay (see Materials and Methods). For simplicity, only two of the four major start sites for the BCL_1 V_H promoter are shown in Fig. 2. We refer only to RSV species that migrated between 55 and 64 bases as specific, since bands below 53 bases also appeared in unincubated (no nucleoside triphosphates [NTPs]) controls. These lower bands seen with the RSV probe are probably due to self-hybridization and self-protection of probe sequences, since the probe by itself also gave rise to these bands after RNase digestion (data not shown). The in vitro transcripts were shown to be correctly initiated by comparing them with BCL_1 and RSV mRNA made in vivo, which also contains multiple start sites. Using this plasmid, we show that nuclear extracts from pre-B cells (SMS-SB and Nalm-6) expressing very low levels of OTF-2 could transcribe the immunoglobulin gene as well as or better than nuclear extract from a Burkitt's lymphoma cell

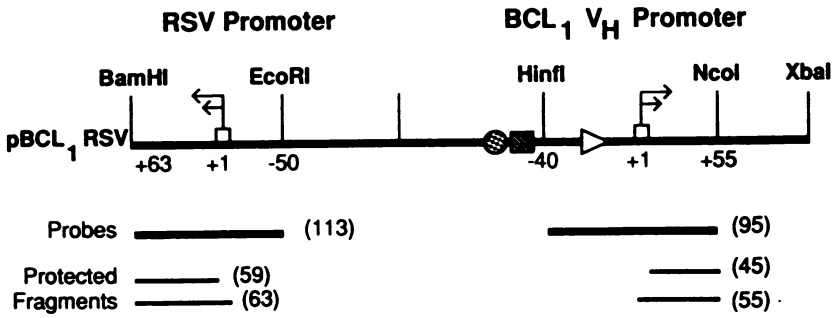

FIG. 2. Template and probes used in the in vitro transcription reaction and RNase protection assay. A 593-bp *Bam*HI-*Xba*I fragment of the expressed heavy-chain immunoglobulin gene from the BCL_1 leukemia cell line was cloned into pUC19. In the opposite orientation a 420-bp *Bam*HI-*Nru*I fragment of the RSV LTR was inserted to make $pBCL_1RSV$. Symbols: □, major start sites of transcription; ●, conserved heptamer; ▨, conserved octamer; △, TATA box. Antisense RNA probes used to detect the in vitro-generated transcripts span the initiation start site of each gene; and their sizes (in bases) are indicated. The protected fragments resulting from the RNase protection assay are also shown, with sizes (in bases) indicated.

line (Namalwa) expressing a relatively high level of OTF-2 (Fig. 3, lanes 1 to 3).

The four nuclear extracts in this experiment contained similar levels of OTF-1-binding activity per milligram of protein in a gel retardation assay (data not shown). Namalwa nuclear extract also contained the additional binding activity associated with OTF-2 at approximately one-half the level of OTF-1 (Fig. 1, lane 2). The fact that Namalwa nuclear extract contained high levels of OTF-1 and OTF-2 yet was no more transcriptionally active than SMS-SB or Nalm-6 nuclear extracts argues against the suggestion (25) that differences in the ability of various cell types to transcribe immunoglobulin genes is a result of a quantitative difference in the amount of total octamer-binding protein present. All

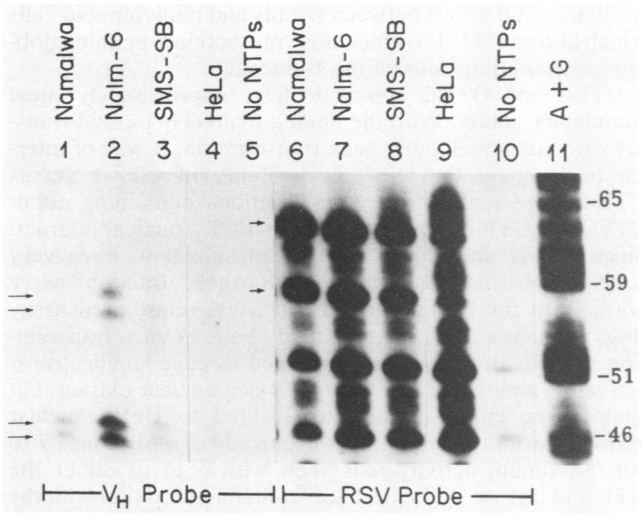

FIG. 3. RNase protection assay of transcripts generated in vitro. In vitro transcripts were made by incubating $pBCL_1RSV$ (Fig. 2) with nuclear extracts made from either Namalwa (lanes 1 and 6), Nalm-6 (lanes 2 and 7), SMS-SB (lanes 3 and 8), or HeLa (lanes 4 and 9) cells. TPs were excluded, and reactions were kept on ice in lanes 5 and 10 as a control for nonspecific bands. Transcripts were detected by using either the BCL_1 V_H (lanes 1 to 5) or RSV (lanes 6 to 10) antisense RNA probe (Fig. 2). These in vitro transcripts were shown to have authentic start sites by comparing them with BCL_1 cellular RNA (see Fig. 4B, lane 10). As a molecular size marker, an A+G reaction was done on an end-labeled, 322-bp fragment from the BCL_1 gene (lane 11); sizes (in nucleotides) are indicated on the right.

cell lines tested transcribe from the control RSV promoter with approximately equal efficiency, whereas transcription from the immunoglobulin promoter is specific for only lymphoid cell nuclear extract. These results support our in vivo data and confirm that the level of OTF-2 in lymphoid cells does not correspond to the ability of these cells to transcribe immunoglobulin heavy-chain genes.

In vitro transcription of immunoglobulin genes is dependent on octamer-binding proteins. Through mutational analysis, it has been shown that an intact octamer is necessary for immunoglobulin promoter activity in vivo and in vitro (8, 12, 28, 41). It has also been shown through transfection experiments that an octamer element placed upstream of a heterologous gene can confer lymphoid specificity (6, 51). To test whether octamer-binding proteins are required for the lymphoid-specific immunoglobulin gene expression seen in our in vitro assay, in vitro transcription competition experiments were performed. Addition of a duplex oligonucleotide containing the octamer sequence to the in vitro transcription reaction specifically inhibited transcription of the immunoglobulin gene, whereas addition of an oligonucleotide containing a mutated octamer did not (Fig. 4A, lanes 1 to 3). This inhibition occurred when either Namalwa, SMS-SB, or Nalm-6 nuclear extract was used. Even the small amount of transcription seen in some experiments by HeLa nuclear extract was inhibited by octamer-containing oligonucleotides (data not shown). The amount of oligonucleotide needed to inhibit transcription roughly correlated with the amount needed to inhibit binding in a gel retardation assay (Fig. 4B, lanes 3 and 4). This inhibition was specific for the immunoglobulin gene, since transcription of the RSV LTR by any of the nuclear extracts was not affected by addition of either oligonucleotide (Fig. 4A, lanes 5 and 6). These results suggest that OTF-1, in the pre-B cells lacking OTF-2, functions through direct octamer binding to transactivate immunoglobulin gene transcription in vitro.

Addition of highly enriched OTF-1 to lymphoid nuclear extracts stimulates immunoglobulin gene transcription. To test more directly whether OTF-1 can function to transactivate immunoglobulin genes, a highly purified preparation of OTF-1, devoid of OTF-2 activity, was added to the in vitro transcription reaction. OTF-1 was purified from both HeLa and SMS-SB cells through a series of chromatography steps, including two passages over an octamer-affinity resin as previously described (12; Materials and Methods). OTF-1 preparations from either cell line appeared identical and

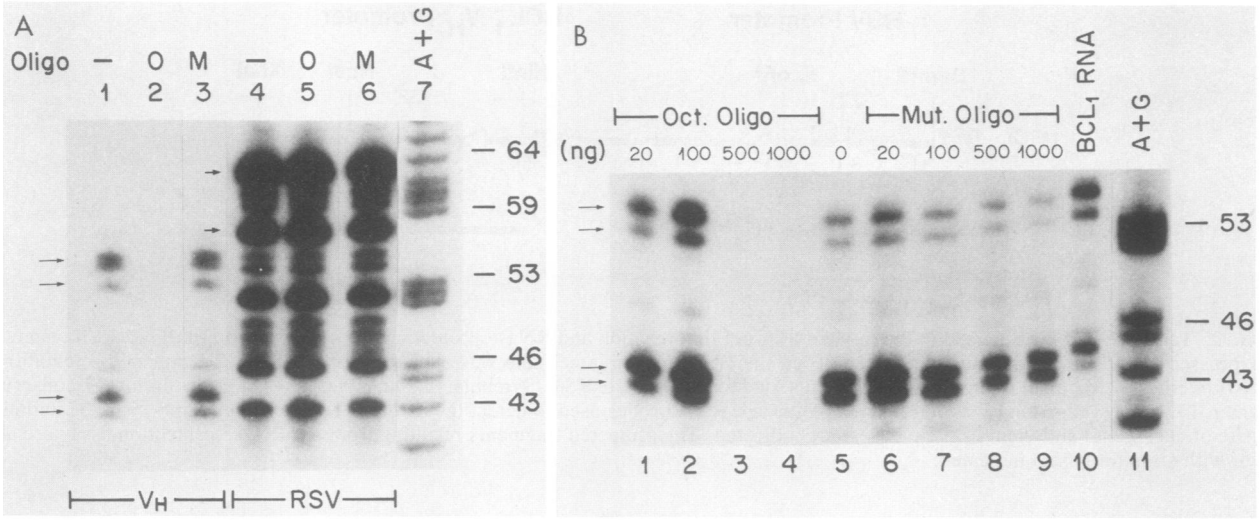

FIG. 4. Competition of in vitro transcription by an octamer-containing duplex oligonucleotide. (A) In vitro transcription reactions were done by incubating $pBCL_1RSV$ with SMS-SB nuclear extract. Transcripts were detected by using either the BCL_1 V_H (lanes 1 to 3) or RSV (lanes 4 to 6) antisense RNA probe. No oligonucleotide (lanes 1 and 4) or 1 μg of duplexed oligonucleotide (20-mer) containing either the octamer (O; lanes 2 and 5) or a mutated form of the octamer (M; lanes 3 and 6) was added to the in vitro transcription reaction. An A+G reaction was done as a molecular size marker (lane 7). (B) In vitro transcription reactions were done using Namalwa nuclear extract and $pBCL_1RSV$. All transcripts were detected by using the BCL_1 V_H probe. No oligonucleotide (lane 5) or increasing amounts of either the octamer-containing oligonucleotide (lanes 1 to 4) or the mutated octamer-containing oligonucleotide (lanes 6 to 9) were added to the reaction. Total RNA (4 μg) isolated from BCL_1 cells was used in the RNase protection assay to demonstrate that in vitro-generated transcripts were correctly initiated (lane 10). An A+G reaction was done as a molecular size marker (lane 11); sizes (in nucleotides) are indicated on the right.

contained three visible bands on a silver-stained SDS-polyacrylamide gel with apparent molecular weights of 100, 92, and 70 kilodaltons (Fig. 5A). Partial amino acid sequence analysis of both the 92- and 70-kilodalton proteins confirmed that neither shared detectable homology with cloned OTF-1 or OTF-2 (L. Carayannopoulos et al., manuscript in preparation). At least one of these proteins, presumably the 100-kilodalton protein by virtue of its size relatedness with cloned OTF-1, gave rise to S1 and S2, shown by others to contain monomeric and dimeric OTF-1, respectively (18, 34, 35). When these OTF-1 preparations from either SMS-SB or HeLa cells were added to SMS-SB nuclear extract, a stimulation of immunoglobulin gene transcription occurred (Fig. 5B and 5C, lanes 2 and 3). When normalized to binding activity, HeLa OTF-1 and SMS-SB OTF-1 gave approximately equal stimulatory activity, suggesting that OTF-1 from lymphoid cells is the same as OTF-1 from nonlymphoid cells. This transactivation was specific for the immunoglobulin gene, since addition of OTF-1 had little effect on RSV LTR transcription (Fig. 5B, lanes 9 and 10). However, when these OTF-1 preparations were added to HeLa nuclear extract, immunoglobulin gene transcription did not appear to be stimulated (Fig. 5B, lanes 5 to 7). The diffuse spots seen in the HeLa lanes were not specific transcripts, since these also could be seen in the unincubated (no NTPs) control. We believe these spots are due to excess tRNA used as carrier in the assay, since when the tRNA amount was reduced or when glycogen was used as a carrier, the spots were not seen. It is possible that these spots may be masking specific transcripts of higher molecular weight, but it is clear that the smaller transcripts were not stimulated by OTF-1 addition. Since we have never observed transcription from some of the start sites but not from the others, we feel that immunoglobulin transcription is not stimulated in this case. This result differs from a previous report in which addition of OTF-1 to HeLa nuclear extract did stimulate immunoglobulin gene transcription (25). These data, using highly purified OTF-1, extend the experiment shown in Fig. 3 indicating that OTF-1 can function to transactivate an immunoglobulin gene in lymphoid nuclear extracts but functions very poorly if at all in HeLa nuclear extract. This finding suggests a qualitative difference between B cells and nonlymphoid cells which allows OTF-1 to efficiently transactivate immunoglobulin genes only in cells of the B lineage.

OTF-1 and OTF-2 appear to have approximately equal stimulatory ability. With the finding that OTF-1 could transactivate immunoglobulin gene transcription, it was of interest to compare the stimulatory ability of OTF-1 versus OTF-2. Size-separated protein fractions containing either OTF-1 or OTF-2 were made from BCL_1 nuclear extract. Since OTF-1 and OTF-2 have been shown to have very similar DNA-binding affinities (40), these fractions were normalized for binding activity in a gel retardation assay (Fig. 6A, lanes 2 and 3) and added to the in vitro transcription system. Both fractions appeared to have similar transactivating ability when added to a B-cell nuclear extract, but again when either fraction was added to HeLa nuclear extract, very little stimulation occurred (Fig. 6B, lanes 7 to 10). Maximum activity was seen with 5 μl of either the OTF-1 (4 μg)- or OTF-2 (2.5 μg)-containing fraction with the B-cell extract. Increasing the amount of OTF in the reaction resulted in a decrease in transcription of the immunoglobulin gene (Fig. 6B; compare lanes 2 and 3 and lanes 4 and 5). This inhibition of transcription with excess OTF has also been noticed by others using different systems (19, 25). Yet even at these excess levels of OTF-1 and OTF-2, immunoglobulin gene transcription was not significantly stimulated when HeLa nuclear extract was used. This finding differs from another report (29) in which a cloned cDNA of a lymphoid-specific octamer-binding protein, presumably OTF-2, was able to activate transcription of a B-cell-specific promoter in HeLa cells in cotransfection experiments. The artificial

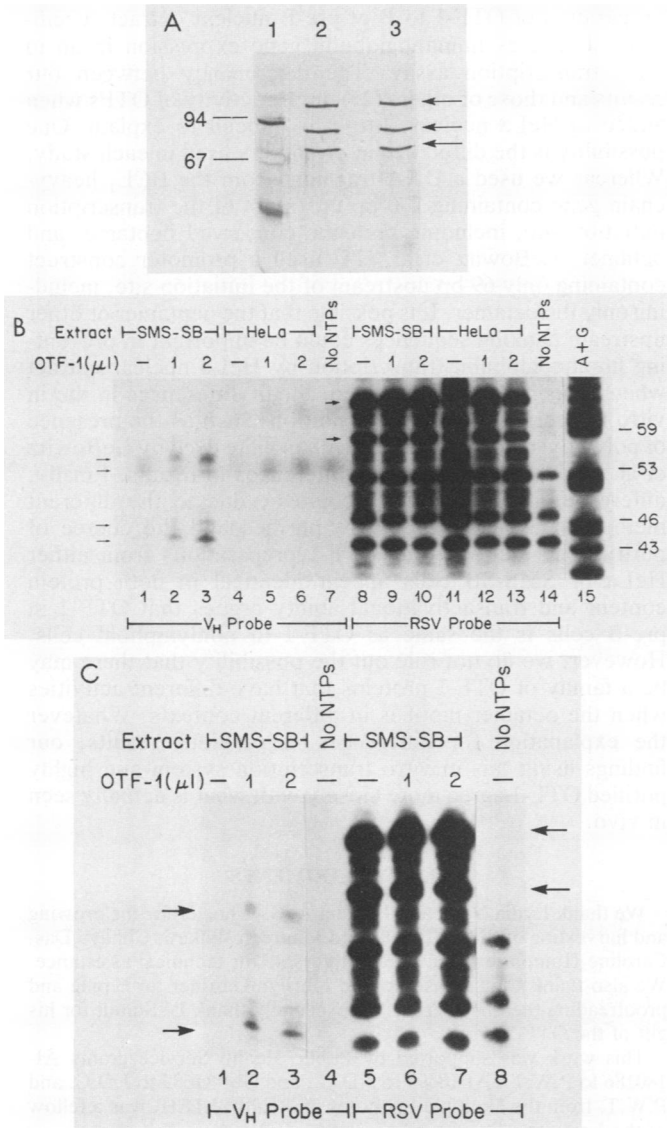

FIG. 5. Addition of affinity-purified OTF-1 preparations to in vitro transcription reactions. (A) Silver-stained 7.5% SDS-polyacrylamide gel of purified OTF-1 preparations from SMS-SB (lane 2) and HeLa (lane 3) cells. Lane 1 contains a low-molecular-size marker mix (Pharmacia), with the sizes of phosphorylase B (94 kDa) and bovine serum albumin (67 kDa) indicated. (B) In vitro transcription reactions were performed by using $pBCL_1RSV$ as the template and reduced amounts of nuclear extract (approximately 200 μg of nuclear protein) from either SMS-SB (lanes 1 to 3 and 8 to 10) or HeLa (lanes 4 to 6 and 11 to 13) cells. No OTF-1 (lanes 1, 4, 8, and 11) or 1 μl (55 ng) (lanes 2, 5, 9, and 12) or 2 μl (110 ng) (lanes 3, 6, 10, and 13) of partially purified OTF-1 from SMS-SB cells was added to the reaction (55 ng of OTF-1 preparation contains approximately the same amount of octamer-binding activity as 25 μg of crude nuclear extract in a gel retardation assay). No NTPs were added, and reactions were kept on ice in lanes 7 and 14 as a control for nonspecific bands. In vitro transcripts were detected by using either the BCL_1 V_H (lanes 1 to 7) or RSV (lanes 8 to 14) antisense RNA probe. An A+G reaction was done as a molecular size marker (lane 15); sizes (in nucleotides) are indicated on the right. (C) No OTF-1 (lanes 1 and 5) or 1 μl (55 ng) (lanes 2 and 6) or 2 μl (110 ng) (lanes 3 and 7) of partially purified OTF-1 from HeLa cells was added to in vitro transcription reactions containing $pBCL_1RSV$ and SMS-SB nuclear extract. No NTPs were added, and reactions were kept on ice in lanes 4 and 8 as a control. In vitro transcripts were detected by using either the BCL_1 V_H (lanes 1 to 4) or RSV (lanes 5 to 8) antisense RNA probe. Arrows indicate specific protected fragments.

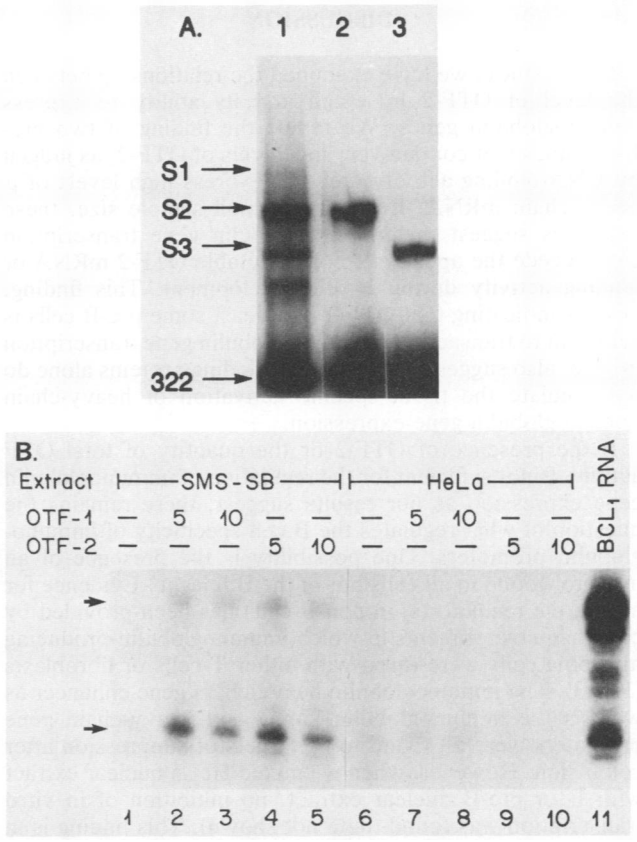

FIG. 6. Analysis of size-separated nuclear protein fractions containing either OTF-1 or OTF-2 by binding and in vitro transcription stimulation. (A) Gel retardation assay of size-separated protein fractions from BCL_1 nuclear extract, using an end-labeled, 322-bp immunoglobulin heavy-chain promoter fragment. Lanes: 1, 5 μl (10 μg) of crude BCL_1 nuclear extract; 2, 5 μl (4 μg) of OTF-1-containing nuclear protein fraction; 3, 5 μl (2.5 μg) of OTF-2-containing nuclear protein fraction. (B) In vitro transcription reactions were done by using $pBCL_1RSV$ as the template and either SMS-SB (lanes 1 to 5) or HeLa (lanes 6 to 10) nuclear extract. The BCL_1 V_H probe was used to detect transcripts in all lanes. OTF-containing fractions were added to each reaction as indicated. Lanes: 1 and 6, no OTF; 2 and 7, 5 μl (4 μg) of OTF-1-containing fraction; 3 and 8, 10 μl (8 μg) of OTF-1-containing fraction; 4 and 9, 5 μl (2.5 μg) of OTF-2-containing fraction; 5 and 10, 10 μl (5 μg) of OTF-2-containing fraction. Total RNA (10 μg) from BCL_1 cells was used in the RNase protection assay to indicate correctly initiated transcripts (lane 11).

promoter construct used in the experiments of Müller et al. (29) did not contain the conserved heptamer (7, 24) found in all immunoglobulin heavy-chain genes, and the distance between the octamer and the β-globin TATA box which was used was different from that found in immunoglobulin genes. These factors may be responsible for the apparent differences in results, we used a fragment of an actual heavy-chain gene containing both the octamer, heptamer, and possibly important flanking sequences in wild-type spatial arrangement. We conclude from these experiments that OTF-1 and OTF-2 appear to have equal transactivation ability when normalized for binding activity. Furthermore, our finding that addition of OTF-2 to HeLa nuclear extract is not sufficient for immunoglobulin gene transcription supports our previous finding that OTF-2 alone does not appear to regulate immunoglobulin tissue specificity, at least at the level of the promoter.

DISCUSSION

In this study, we have examined the relationship between the level of OTF-2 in a cell and its ability to express immunoglobulin genes. We report the finding of two pre-B-cell lines that contain very low levels of OTF-2, as judged by DNA-binding activity, yet still express high levels of μ heavy-chain mRNA. Despite the small sample size, these examples suggest that immunoglobulin gene transcription can precede the appearance of detectable OTF-2 mRNA or binding activity during B-cell development. This finding, besides indicating that OTF-1 in at least some pre-B cells is sufficient to transactivate immunoglobulin gene transcription in vivo, also suggests that octamer-binding proteins alone do not regulate the tissue-specific activation of heavy-chain immunoglobulin gene expression.

If the presence of OTF-2 or the quantity of total OTF activity is not sufficient for the regulation of immunoglobulin gene expression as our results suggest, there remains the question of what regulates the B-cell specificity of immunoglobulin promoters. One possibility is the presence of an inhibitor found in all cells not of the B lineage. Evidence for a negative regulator(s) in non-B cells has been provided by cell fusion experiments in which immunoglobulin-producing myeloma cells were fused with either T cells or fibroblasts (17, 53). The immunoglobulin heavy-chain gene enhancer as well as the immunoglobulin kappa and heavy-chain gene promoters were all found to be targets of suppression after cell fusion. However, when we mixed HeLa nuclear extract with B or pre-B nuclear extract, no inhibition of in vitro transcription was found (data not shown). This finding is in agreement with those of others (25) and argues against the presence of a preexisting dominant repressor in nonlymphoid cell nuclear extract which acts directly on immunoglobulin promoters.

Another possibility is that an additional positive regulatory factor(s) found only in B cells is required for efficient expression of immunoglobulin genes. This factor(s) would likely be non-DNA binding or weakly DNA binding and exert its activity primarily through protein-protein interactions. An example of just such a protein is Vmw65, a virion component of herpes simplex virus responsible for the transcriptional transactivation of viral immediate-early genes. A *cis*-acting sequence, TAATGARAT (R = purine), is found at least once in all of these immediate-early gene promoters and is required for Vmw65 transactivation (26). Vmw65, however, does not bind DNA directly and has recently been shown to require complex formation with an octamer-binding protein very similar to, if not identical with, OTF-1 in order to exert its activity (11, 19, 30, 31, 36, 49). The octamer-binding protein binds the TAATGARAT sequence but cannot transactivate these promoters without the presence of Vmw65. Additional flanking sequences, besides the core octamer-binding site, are also needed for OTF-Vmw65 complex formation and transactivation. The presence of a Vmw65-like protein in B cells which interacts with OTF-1 and OTF-2 as well as important flanking sequences found in immunoglobulin promoters could explain our results. Other regulatory factors associating with OTFs could also explain the additional wide range of activities such as cell cycle regulation of the histone H2b gene or adenovirus DNA replication requiring an intact octamer sequence.

In this report, we document that the level of OTF-2 in lymphoid cells does not correspond to the ability of these cells to express immunoglobulin genes in vivo or in vitro. Furthermore, we find that addition of a highly enriched preparation of OTF-1 to B or pre-B nuclear extract specifically stimulates immunoglobulin gene expression in an in vitro transcription assay. The discrepancy between our results and those of others (25) on the activity of OTFs when added to HeLa nuclear extract is difficult to explain. One possibility is the difference in promoters used in each study. Whereas we used a DNA fragment from the BCL_1 heavy-chain gene containing 270 bp upstream of the transcription initiation site, including both the conserved heptamer and octamer, LeBowitz et al. (25) used a promoter construct containing only 69 bp upstream of the initiation site, including only the octamer. It is possible that the heptamer or other upstream flanking sequences could be important in preventing immunoglobulin transcription by HeLa nuclear extract when exogenous OTF is added. Small differences in the in vitro transcription reaction conditions such as the presence of polyvinyl alcohol or creatine phosphate used by LeBowitz et al. might also account for differences in results. Finally, differences in OTF activity could be due to the different method by which OTFs were purified and the degree of purity. The fact that our OTF-1 preparations from either HeLa or SMS-SB cells appear identical in both protein content and transactivational ability argues that OTF-1 in pre-B cells is the same as OTF-1 in nonlymphoid cells. However, we do not rule out the possibility that there may be a family of OTF-1 proteins that have different activities when the octamer motif is in different contexts. Whatever the explanation for differences in previous results, our findings using this in vitro transcription system and highly purified OTF-1 agree more closely with what is actually seen in vivo.

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