Viral Interleukin 10 Is Critical for the Induction of B Cell Growth Transformation by Epstein-Barr Virus

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Summary

We have used an efficient cDNA subtraction library procedure to identify newly induced genes in human B lymphocytes infected for 6 h with Epstein-Barr virus (EBV). Among the genes identified by automated sequencing of a random subset of clones from this library, one coded the EBV BCRF1 open reading frame, which specifies the viral interleukin 10 gene (vIL-10). This molecule is highly homologous to human (h)IL-10 and was previously thought to represent a "late" viral gene expressed only during the lytic phase of virus replication. Using gene amplification by reverse transcriptase polymerase chain reaction of B cell RNA obtained at varying times after infection, we detected vIL-10 expression within a few hours of EBV infection, followed, 20–30 h later by expression of hIL-10. Expression of both genes continued beyond the initial transformation phase (5–10 d) and was present in all transformed cell lines tested. When added at the time of viral infection, antisense (but not sense) oligonucleotides for vIL-10 mRNA (cytosolic half-life, ~6 h) prevented subsequent B cell transformation. The antisense effect was highly specific, leaving the expression levels of other transformation-related genes intact. Addition of exogenous (h)IL-10 rescued the transformation process in antisense-treated cells. Our observations establish vIL-10 as a new latency gene with a directly transformation-prerequisite function.

EBV is a highly prevalent herpes virus associated with a growing number of lymphatic and epithelial malignancies (1–9). Lymphocytes (mainly of B cell lineage) that express the CD21 EBV receptor (10) are efficiently growth transformed by the virus, and such cells develop into fatal lymphomas in susceptible hosts (11–13). EBV is maintained in its target cell as a nonintegrated, 180-kb episomal plasmid. In growth-transformed B cells the virus stays latent and transformed B cells enter lytic cycle with production of infectious virus progeny rarely at best (14). Latency of stable, growth-transformed B lymphoblasts is characterized by the expression of viral proteins, Epstein-Barr nuclear antigens (EBNAs)[1] -1, -2, -3a, b, and c, latent membrane protein (LMP-1), and terminal protein (TP)2a,b (LMP-2A/B) (15).

Binding of EBV to the CD21 cell surface receptor starts two series of events that initially proceed independently although both are critical for subsequent growth transformation. A cellular activation cascade of postreceptor binding events includes calcium and proton currents and the induction of tyrosine kinase and stress proteins (16–18). Interference with any of these events prevents transformation, generating viable, principally transformable cells that harbor competent virus without undergoing growth transformation (16).

[1] *Abbreviations used in this paper:* ds, double stranded; EBNA, Epstein-Barr nuclear antigen; h, human; RNA^{ind}, EBV-induced B cell RNA; RNA^{com}, common B cell RNA; RT, reverse transcriptase; v, viral.

The second series of events, viral latency gene expression, immediately follows circularization and translocation of the linear virus genome to the nucleus. Transcripts of at least four latency genes (EBNA-1 and -2, and LMP-1 and -2) are detectable within 30 min of infection (16, 19). Transcription is independent of the cellular activation cascade but intimately regulated; the transient suppression of any one of these genes by antisense reagents aborts expression of all others and prevents subsequent growth transformation (19).

It is still unclear how and when these two chains of postinfection sequelae combine into the events that initiate transition to and then sustain the growth-transformed phenotype. We have argued that these events are most likely associated with the transient or permanent activation of genes devoted to the regulation of cell growth, and we chose a subtractive molecular approach as a strategy for their identification. Here we describe the construction of a cDNA library highly enriched for genes expressed in B cells infected with EBV for 6 h, and report IL-10 as the first transformation prerequisite gene identified by our strategy.

Results of a first, random sampling of clones obtained after subtraction were unexpected. Sequencing identified the viral BCRF1 gene, a sequence highly homologous to the human IL-10 gene (20). The BCRF1 (viral[v] IL-10) gene had been characterized as a "late" viral gene expressed during the lytic phase of virus replication (21) and it shares most functions

with its human homologue (22). This includes the suppression of IL-2 and IFN-γ production as well as potent B cell growth-promoting activity (23–28). We now demonstrate that vIL-10 is expressed early during the transformation process, preceding the expression of cellular human (h)IL-10 by 20–30 h, and that both continue to be expressed in established EBV-transformed cell lines. As the transient suppression of vIL-10 expression by short-lived antisense reagents aborted subsequent transformation, our data establish vIL-10 as a new, bona fide latency gene whose function is critical for at least the initiation and perhaps the maintenance of the transformed phenotype.

Materials and Methods

The preparation of purified, largely resting B lymphocytes from tonsil and of B95-8 strain EBV transformation as well as cell culture conditions have been described previously (17). B cells were infected with virus (10^8 particles/3 × 10^6 cells) and total RNA was prepared by the guanidine thiocyanate method (29) either immediately (common B cell RNA [RNA^{com}]) or after 6 h of culture (EBV-induced B cell RNA [RNA^{ind}]). Poly(A) RNA was purified using oligotex-dT30 latex beads (gift from Japan Rosch Inc., Tokyo, Japan), which carries covalently surface-bound oligonucleotide chains of the structure $d(G)_{10}d(T)_{30}$ (30). Total RNA (500 μg/tube) was mixed with 1% (wt/vol) of the oligotex-dT30 beads in 1 ml elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS), incubated (65°C for 5 min), and cooled. After addition of NaCl (to 100 mM), the beads were held at 37°C for 10 min, spun, washed twice (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS, 0.1 M NaCl), and resuspended in 400 μl of water. Bound mRNA was eluted at 65°C into the supernatant and precipitated with 2 vol of cold ethanol.

Solid-Phase $cDNA^{com}$ Library. After hybridization of purified poly(A) RNA (20 μg) from resting B cells ($mRNA^{com}$) to oligotex beads, covalently bound cDNA was synthesized with AMV reverse transcriptase (RT) (1,000 U, 42°C/45 min; BRL, Burlington, Ontario, Canada), exploiting as primers the covalent $d(T)_{30}$ tails of the beads resuspended in reaction buffer (100 mM Tris-HCl, pH 8.3, 100 mM KCl, 10 mM $MgCl_2$, 2 mM each dNTP, 20 mM 2-ME, 500 U/ml of RNasin, 500 μg/ml BSA) (30). After heat denaturation (92°C/5 min), the $cDNA^{com}$-oligotex-dT30 conjugate was spun and washed to remove RNA, and resuspended in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Subtraction of $mRNA^{ind}$. 1 μg of $mRNA^{ind}$ was incubated (10 min/55°C) with 5 μg oligo-dT_{12-18}, and the $cDNA^{com}$-oligotex conjugate (100 μl) was incubated with 50 μg of oligo-$dA_{30}dG_{10}$. Both were then mixed and held at 55°C for 10 min in a total reaction volume of 500 μl elution buffer with 0.1 M NaCl. Reaction tubes were spun, supernatants recovered, and the pellets resuspended in 500 μl of TE, denatured (75°C/5 min), and washed to remove bound RNA to regenerate the single-stranded (ss)-$cDNA^{com}$ library for subsequent subtraction cycles. The subtraction efficiency during several cycles was measured by densitometry of slot blots hybridized to radiolabeled β-actin (common cellular) and EBNA-2 (viral latency gene) probes, controlled by RT-PCR amplification with β-actin and EBNA-2 oligonucleotides (31).

After four subtraction cycles of $mRNA^{ind}$, ss-cDNA was synthesized (37°C/60 min) with Moloney murine leukemia virus (MMLV) RT (200 U; BRL) in 50 mM Tris-HCl (pH 8.3) containing 75 mM KCl, 3 mM $MgCl_2$, 1 mM each dNTP, 10 mM DTT, and 80 U RNasin followed by generation of second-strand cDNA (16 h/14°C) in the presence of 20 U/ml RNase H, 500 U/ml *Escherichia coli* DNA polymerase I, and 100 U/ml *E. coli* DNA ligase (New England Biolabs, Mississauga, Ontario). After generation of blunt ends (37°C/45 min in 100 ng/ml RNase A, 50 U/ml RNase H, 250 U/ml *E. coli* DNA ligase, and 100 U/ml T_4 DNA polymerase), EcoRI adapters were added and 1–10-kb double-stranded (ds)-cDNA fragments were recovered from a 1% low melting agarose gel. Recovered ds-cDNA fragments were ligated to EcoRI-digested λgt10 arms and then packaged into λ particles (Amersham, Oakville, Ontario). After phage infection of *E. coli* NM514 and overnight culture on H-medium plates, plaques were transferred onto Hybond-N+ (Amersham Corp., Arlington Heights, IL) and hybridized to random-primed ^{32}P-cDNA derived from: (*a*) $mRNA^{com}$; (*b*) $mRNA^{ind}$; and (*c*) a series of end-labeled oligonucleotides derived from EBV and cellular genes (see below). A normal tonsil (λgt-11) library was obtained from American Type Culture Collection (Rockville, MD) (37546) as a control.

DNA Sequencing. DNA sequencing by primer extension was carried out with fluoresceinated λgt10 forward (5'-FAM-AGCAAGTTCAGCCTGGTTAAG) and reverse (5'-FAM-CTTATGAGTATTTCTTCCAGGGTA) primers using the dideoxy-AutoRead™ Sequencing kit (Pharmacia, Mississauga, Ontario) as instructed. Terminated samples were analyzed on an automated laser fluorescence (A.L.F.™) automated DNA sequencer following protocols of the manufacturer (Pharmacia).

PCR. Standard, 30-cycle PCR used primers from the EBV and cellular gene sequences previously described (8) in addition to reagents for amplification of vIL-10 (32) and hIL-10 (22): vIL-10-9681, 5'-CGAAGGTTAGTGGTCACTCT; vIL-10-10186(A), 5'-CACCTGGCTTTAATTGTCATG; hIL-10-940, 5'-CGCTTTCTAGCTGTTGAGCT; hIL-10-1400(A), 5'-CACTGCAACTTCCATCTCCT. Internal oligonucleotide probes were vIL-10-9921, 5'-TACCTGGAGGAAGTCATGCC; and hIL-10-1277(A), 5'-CACCATGTTGACCAGGCTGGTT. Numbers indicate the 5' position in the EBV genomic sequence (33) and hIL-10 (22). RT-PCR amplifications were controlled by omission of RT; these reactions as well as template-free water controls were negative. Oligonucleotides were synthesized on a GeneAssembler Plus™ instrument according to supplied protocols (Pharmacia).

B Cell Transformation Studies. The transformation of fresh tonsillar B cells was examined as described (17). Briefly, purified B cells (3–5 × 10^6) were exposed to 10^8 B95-8 strain EBV particles, incubated (90 min/37°C) in HL/1 medium (Ventrex, Portland, ME) until distributed into microcultures (10^5 cells/well). Where indicated, fresh lymphocytes were first loaded with 20mer sense or antisense oligonucleotides (1 μg) during a 2-h incubation (17). In some experiments 10 U/well of recombinant hIL-10 (gift from Dr. K. Moore, DNAX, Palo Alto, CA) was added at the beginning of culture. Cultures were maintained until supernatants were removed 2 wk later. Transformation was assessed as clonal expansion, measuring cell growth with an automated, fluorescence-based procedure (16), or by measurement of secreted Ig, using a particle concentration fluorescence immunoassay technique (17). We previously found good correlation between both approaches (18).

Results

Subtraction Library. We used the detection of a housekeeping gene (β-actin) and of IgM (Cμ heavy chain gene) to assess the efficiency of our cDNA subtraction procedure. Densitometer scans of RNA slot blots hybridized to a β-actin

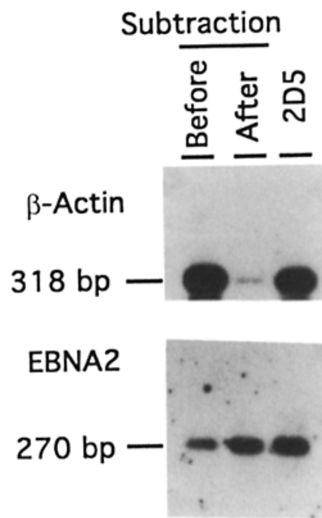

Figure 1. PCR amplification of β-actin and EBNA-2 fragments. Equal volumes of ds-cDNA from before and after subtraction were amplified with β-actin or EBNA-2 primers, and the PCR product was hybridized to ^{32}P end-labeled internal reporter probes. The EBV-transformed B cell line 2D5 was generated in our laboratory and served as a control.

probe showed a 50% reduction of signal after the first subtraction cycle and >90% reduction after the second. Thereafter the signal was lost. Northern gel hybridization detected β-actin transcripts in $mRNA^{com}$ but not in 4× subtracted $mRNA^{ind}$, and we therefore used RT-PCR (30 cycles) to detect less prevalent species (Fig. 1). β-Actin sequences were amplified in both RNA samples but only faint hybridization signals were obtained with PCR products of reverse-transcribed $mRNA^{ind}$.

EBV latency gene transcripts are not detectable by Northern blot hybridization until several days after infection (34), although latency gene transcription of at least four of the latency genes begins as early as 30 min postinfection and is a critical transformation prerequisite (19). Transcripts of EBNA-2 (Fig. 1), EBNA-1, and the LMPs (not shown) were easily detected by RT-PCR of $mRNA^{ind}$, both before and after 4× subtraction. These data suggested a reasonable efficiency of the subtraction process: we estimate that 0.5% of common genes in RNA^{com} remain after 4× subtraction, although these species make up the vast majority of transcripts in RNA^{com}. On the other hand, ∼70% of transcripts specific for RNA^{ind} were still present after subtraction.

This was confirmed after cloning of subtracted ds-cDNA. We obtained a total of ∼2 × 10^5 recombinant phage. Replicate nylon membrane lifts from plates containing 1,600 recombinant clones each were hybridized with randomly primed and labeled $mRNA^{com}$ or $mRNA^{ind}$ (Fig. 2). About 15% of phages hybridized with the RNA^{com} "probe," while 78% hybridized with the RNA^{ind} "probe" (Table 1). 1 out of 3,200 phages hybridized with an IgM probe and none hybridized with β-actin. We recently reported that EBV induces the cellular genes p56-*lck*, *hsp*70, and *hsp*90 early in the transformation process (17, 18). Recombinant clones hybridizing to lck and hsp70 probes were identified at a frequency of 0.25 and 1.3%, respectively. When we used similar conditions to analyze a normal tonsil cDNA library in λgt-11 (3,000 recombinant phage) as a control, we found 0.53% of clones hybridizing with the IgM and 0.26% with the β-actin probes. None of the plaques hybridized to the lck probe and one plaque hybridized to the hsp70 probe.

Gene Induction after EBV Infection. The above data indicated that the subtraction library generated from RNA^{ind} showed reasonable enrichment for transcripts induced in fresh human B lymphocytes within 6 h of EBV infection. We randomly chose 21 phages grown at very low density, amplified, and used PCR with flanking, λgt10-derived primers to generate sufficient DNA template for sequence analysis. Fluorescein end-labeled primers were then used for direct dideoxy sequencing and subsequent analysis on the A.L.F.™ automated DNA sequencer.

In six clones no PCR product was generated, and in four clones the fragment size was <200 bp. One clone each contained EBNA-1 and EBNA-2 sequences, one contained a partial coding sequence of CD19, and another of glucose-6-phosphate reductase. CD19 transcripts are of potential interest as

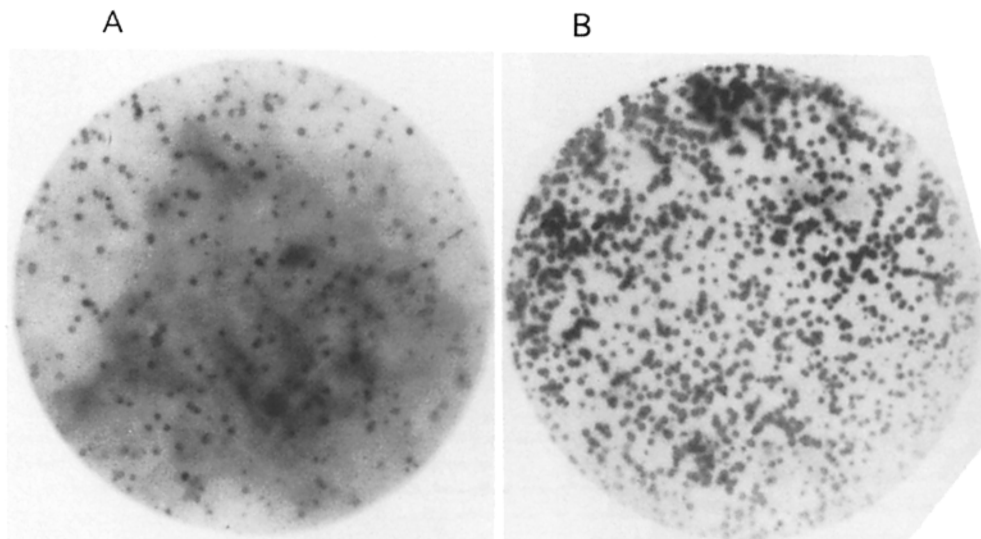

Figure 2. Plaque hybridization of a subtraction library. Approximately 1,600 recombinant phages each were plated on two 137-mm dishes. Replicate lifts on nylon membrane filters were probed with randomly primed, ^{32}P-labeled cDNA made from: (*A*) $mRNA^{com}$ or (*B*) $mRNA^{ind}$.

Table 1. *Plaque Hybridization of Subtraction and Standard B Cell Libraries*

Library	Total plaques*	Probes			
		cDNA^com	cDNA^ind	IgM	β-Actin
Subtraction	3,200	486	2,500	1	0
Tonsil	3,000	2,800	ND	16	8

Subsets of two cDNA λ libraries (see text) were hybridized with radiolabeled cDNA obtained from purified, fresh human B lymphocytes that had been infected with EBV for 0 (cDNA^com) or 6 h (cDNA^ind). Labeled probes for the human Cμ heavy chain (IgM) or the human β-actin gene were used in addition. Numbers refer to plaques that hybridized to the respective probes.
* Combined data from two independent plates.

this molecule plays a dual role in B cell function, triggering positive or negative signals depending on preceding or coactivation events (35). Somewhat unexpectedly, three clones contained sequence stretches highly homologous (82–87%) to different cytochrome p450 genes: to our knowledge, this enzyme system has not previously been associated with B cell transformation. The full sequence characterization of these clones is in progress. However, we were surprised to obtain one sequence identical to the BCRF-1 reading frame in the EBV genome. This is a late gene expressed during the lytic phase of virus replication and therefore not expected in latently infected B lymphocytes. BCRF1/vIL-10 shows a high degree of homology to the hIL-10 gene, with which it shares most, albeit not all, functions (reviewed in reference 22).

Concerned that the BCRF1 clone might represent a genomic, EBV-derived clone, or that by accident the B cell donor was undergoing an unrecognized, acute EBV infection or EBV-driven lymphoproliferative disease (12), we decided to further pursue this observation. Although we had previously (as expected) found no evidence for the expression of late EBV genes early during transformation (17, 19), we now used PCR and found that neither the original RNA^com nor the RNA^ind or the subtracted library contained sequences coding for gp350/220, a late viral (envelope) protein (33) (data not shown). As well, we used several previously designed oligonucleotide probes derived from intron regions in the EBV genome (19) and found no phages that hybridized among 3,200 tested. In contrast, 12 recombinant phages hybridized to a vIL-10 oligonucleotide probe.

vIL-10 Expression during B Cell Transformation. PCR primers and probes were designed for the differential amplification and hybridization of either h- (cellular) or vIL-10. Only the latter system amplified a correctly sized fragment from a vIL-10 cDNA (22) (gift from K. W. Moore, DNAX Research Institute, Inc.), while only the former amplified a hIL-10 sequence from mitogen-activated blood lymphocytes (not shown). Purified B cells were infected with EBV, RNA was prepared 2–120 h after infection, and amplified with vIL-10, hIL-10, or β-actin RT-PCR systems.

As shown in Fig. 3, freshly EBV-infected human B cells begin expression of vIL-10 within 4–6 h of infection. Consistently, no transcription of the cellular hIL-10 gene was detectable 12–18 h after infection, but easily detectable levels of hIL-10 transcripts are present at and beyond 1 d into the transformation process. Comparable amplifications were observed for cellular β-actin in all samples.

Control reactions were performed with a number of different cell lines. The EBV-negative T cell line Jurkat (17) did not express vIL-10 or hIL-10. In contrast, all of nine EBV-transformed cell lines generated previously in our laboratory expressed both vIL-10 and hIL-10 (e.g., 2D5; Fig. 3). vIL-10, but not hIL-10, transcripts were amplified in RNA from the B95 (marmoset) cell line (33), consistent with a polymorphic sequence of the cellular gene in this species. The EBV-producing human P3HR1 line was consistently positive for vIL-10 and hIL-10 expression. Taken together, our observations characterize vIL-10 as bona fide EBV latency gene that is regularly expressed before and then in addition to hIL-10 in EBV-infected B lineage cells.

vIL-10 Functions in the Transformation Process. We have previously used antisense oligonucleotides to determine if genes

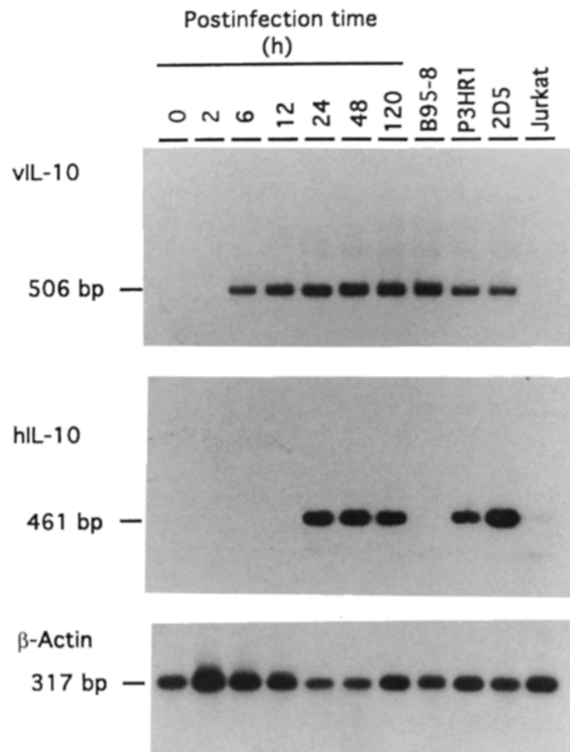

Figure 3. Expression of vIL-10 and hIL-10 in freshly EBV-infected B lymphocytes and in several control cell lines. Poly(A) RNA was prepared at various times after infection of fresh B cells or from exponentially growing cell lines. RT-PCR used primer pairs that distinguish the two IL-10 species. PCR products were separated and probed with ^{32}P end-labeled internal oligonucleotides.

Table 2. *Oligonucleotide Sequences*

Name*		Sequence
vIL-10	– 20	TAGGCCTGCACACCTTAGGT
vIL-10 (A)	– 1	ACCTAAGGTGTGCAGGCCTA
vIL-10	61	TGTGGAGGTACAGACCAATG
vIL-10 (A)	80	CATTGGTCTGTACCTCCACA
vIL-10	502	AAAGCCAGGTGATAATTCCA
vIL-10 (A)	521	TGGAATTATCACCTGGCTTT
hIL-10	31	ATGCACAGCTCAGCACTGCT
hIL-10 (A)	50	AGCAGTGCTGAGCTGTGCAT
hIL-10	112	AACAGCTGCACCCACTTCCC
hIL-10 (A)	131	GGGAAGTGGGTGCAGCTGTT
hIL-10	921	TCCATTCCAAGCCTGACCAC
hIL-10 (A)	940	GTGGTCAGGCTTGGAATGGA

* A, antisense sequences. Numbers represent the 5' position of oligonucleotides on the sense strand.

expressed early after EBV infection played a direct role in the transformation process (17, 19). The same strategy was used to determine if vIL-10 subserved any role in the transformation process, not a foregone conclusion since strong hIL-10 expression is observed within 1 d of virus infection. Conventional sense and antisense oligonucleotides were synthesized from h- and vIL-10 regions identified in Table 2. Aliquots of fresh B lymphocytes were loaded with one of these reagents each, infected with EBV, and transformation was measured 2 wk later (17).

As shown in Fig. 4, vIL-10 antisense, but not sense, oligonucleotides reduced transformation to essentially background levels. In the experiment shown the Ig secretion of antisense oligonucleotide-treated cells approached that of unstimulated B cells. Similar results were obtained when clonal expansion was assessed by measurement of cell growth. The half-life time of these oligonucleotides is ~6 h (17, 19), and it was not surprising that reagents directed at hIL-10 transcripts were ineffective, as our above results determined that hIL-10 is expressed a full 1 d later than vIL-10.

To determine the mode of action of vIL-10 antisense oligonucleotides, we examined levels of vIL-10 transcripts. Freshly prepared B lymphocytes were incubated with sense (vIL-10 502) or antisense (vIL-10 [A] 521) oligonucleotides as above. RNA was extracted from 6-h postinfection cells and treated with DNaseI. ss-cDNA was synthesized with oligo(dT) primer from 500 ng of bulk RNA, and ~5 ng of ss-cDNA was amplified by RT-PCR (30 cycles). vIL-10 gene transcription was dramatically inhibited in RNA from antisense-treated cells while there was no effect of this treatment on β-actin transcription levels (Fig. 5). The antisense effect was specific, since levels of EBNA-2 transcripts, which show a comparable, low abundance as vIL-10 transcripts, were unaffected by vIL-10 antisense oligonucleotides. Titrations of template concentrations indicated that the amplification of β-actin fragments was well below saturation level in our PCR system (data not shown).

While sense/antisense oligonucleotide effects showed clearcut sequence/strand specificity, we tested a number of additional reagents. Sense and antisense oligonucleotides were added at the time of EBV infection to cultures of purified B lymphocytes. Targeting of several coding regions of IgG, IgM, or IgA heavy chain transcripts did not affect transfor-

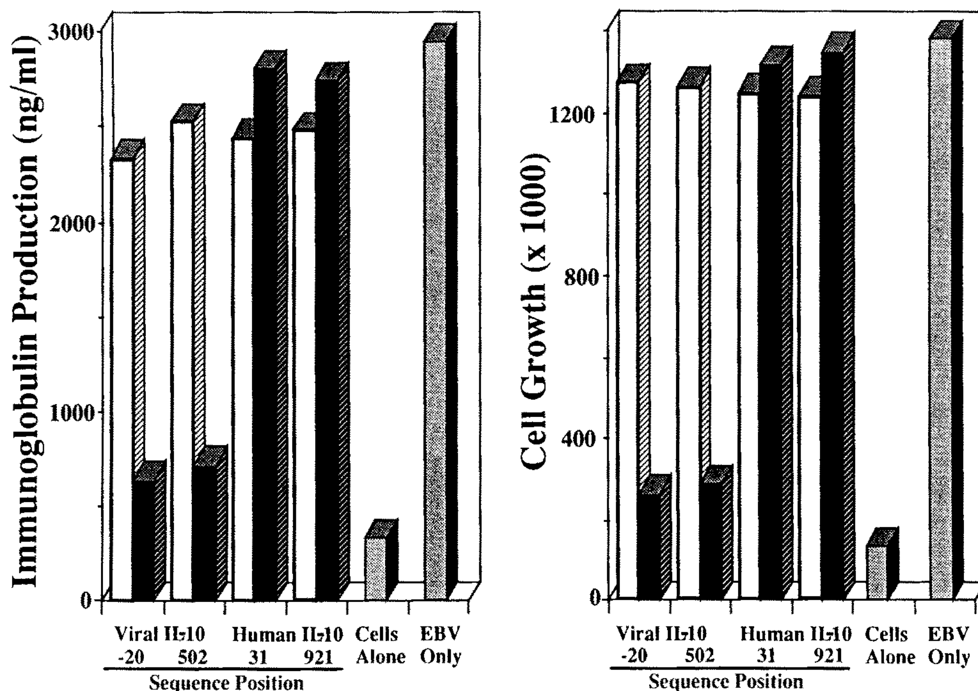

Figure 4. vIL-10 antisense oligonucleotides abort transformation. Purified fresh B lymphocytes were loaded with various sense (□) or antisense (■) oligonucleotides before EBV infection and culture. The 5' positions of these oligonucleotides on the sense strand of the respective vIL-10 or hIL-10 sequences are indicated below the graph (see Table 1). Controls (▩) received EBV alone or no virus. Transformation was measured as virus-induced Ig secretion (*left*) or cell growth (viable cells/ml; *right*) 2 wk later.

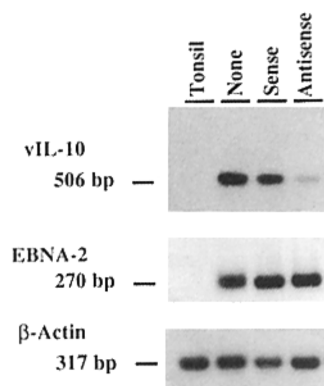

Figure 5. Effect of antisense oligonucleotides on transcription levels of vIL-10, EBNA-2, and β-actin. Purified fresh B lymphocytes were incubated with sense (vIL-10 502) or antisense (vIL-10 [A] 521) oligonucleotides as before. RNA was prepared from 6-h postinfection cells for RT-PCR. Amplification products were separated and probed as before.

mation rates, nor the EBV-induced Ig secretion detected at the end of culture. Similarly, sense and antisense oligonucleotides derived from the β-actin sequence had no effect on EBV-induced transformation, nor did targeting of the late viral gene gp350/220 (data not shown).

We then asked whether the observed vIL-10 requirement in the transformation process had an endogenous mode of action, whether it functioned in autocrine fashion through receptor interaction, and whether the effect showed biospecificity. These experiments were done using recombinant hIL-10, as the viral product was not available to us. However, vIL-10 and hIL-10 share most functional activities, including receptor binding (22, 28, 36). As shown in Table 3, the disrupted transformation process in antisense-treated cells could be fully rescued by addition of exogenous (h)IL10. The vIL-10 antisense effects therefore showed the expected biological specificity and this function appears to resemble an autocrine mechanism likely involving receptor interaction. An effect of exogenous IL-10 was only observed in the vIL-10 antisense-treated cells, and the IL-10 mediated rescue of transformation did not reflect a general growth-promoting effect. Taken together, these findings establish a critical role for vIL-10 transcription very early in the transformation process.

Discussion

In this report we have used an effective procedure for the generation of a subtraction cDNA library towards the identification of a gene, newly discovered to be a transformation prerequisite. The subtraction library contained vIL-10 transcripts and subsequent functional studies showed clearly that vIL-10 is not a late viral gene expressed only during the lytic phase of virus replication, but represents a true latency gene (37), expressed very early in the transformation process as well as in latently infected cells with stable, growth-transformed phenotype (38).

vIL-10, i.e., the BCRF1 open reading frame in the EBV genome, was shown in 1990 (20) to contain a sequence highly homologous to hIL-10. This was an intriguing finding as IL-10 has functions highly consistent with survival and propagation of a B-lymphotropic transforming virus. Viewed as the agent that directs immune responses towards either mainly cellular (T lymphocyte mediated) or humoral effector principles, IL-10 directly and through accessory cells (25) interferes with IL-2 and IFN-γ production (26, 27) but acts as a potent (28) B cell growth factor (39, 40), and it may be the active principle behind apparent suppressor cell phenomena (24). It was primarily IL-10's ability to interfere with IFN-γ production and T effector cell generation that painted an appealing scenario for the escape of a virus-infected B cell from T cell surveillance, in particular after it was determined that vIL-10 shared many relevant functions with the full human molecule (22).

Our data do not detract from the possible role of IL-10 in the escape of EBV-infected B cells from T cell surveillance, and given the tremendous potential of EBV-infected cells to rapidly develop into aggressive lymphomas (11, 12, 41–43), this remains an important area of research. However, our findings add a new dimension to IL-10 and its viral homologue, since vIL-10 now appears as a true latency gene with a specific, critical role in the growth transformation process itself.

To study genes that play critical roles during the initial transformation events, we have previously used short-lived, conventional sense and antisense oligonucleotides with half-lives of ~6 h (17). These experiments showed that the transient impairment of specific viral and cellular transcripts can effectively abort subsequent transformation, even though competent virus is harbored in perfectly transformable, viable cells. This strategy is able to delineate critically timed gene expression events, and we demonstrated the closely interactive, con-

Table 3. *Rescue of Antisense-inhibited Transformation by Exogenous IL-10*

			EBV + vIL-10 oligonucleotides				+ hIL-10 oligonucleotides	
Cytokine	Unstim.	EBV	−20	(A) −1	502	(A) 521	112	(A) 121
None	290	1,340	1,116	458	1,118	523	919	1,023
hIL-10	320	1,159	1,212	1,132	1,233	1,140	1,042	1,058

Cell growth (× 10^3) of EBV-infected cells after 12 d of incubation. Sense or antisense (A) oligonucleotides derived from the v- or hIL-10 coding sequence (see Table 2) were added as indicated. Cultures received either no or 10 U/ml of recombinant, hIL-10. Unstim., no EBV added.

certed fashion that characterizes the regulation of gene expression for four such genes (19).

The exact functions of those viral latency genes that were identified as critical in the early transformation process are largely but not entirely uncertain. EBNA-1 acts in *cis* by binding the virus' latent origin of replication (ori-P) to maintain the virus circular genome in its episomal locale (44). LMP-1, at least in some target cells (45, 46), activates, in *trans*, the cellular protooncogene, *bcl*-2 (47), which may prevent apoptosis in cells susceptible to this process (48). Since essentially all infected B cells undergo early virus-induced activation events but <10% reach stable transformation, there must be B cell–inherent protective mechanisms that interfere with transformation. Apoptosis is a candidate mechanism in this process, perhaps as a result of post-EBV receptor binding events that represent a typical, if to considerable extent, EBV-specific activation cascade with calcium and proton currents as well as tyrosine kinase and stress protein induction (16–18).

The addition of IL-10 to the emerging map of early steps in the transformation process is likely important because of the direct B cell growth-promoting function of the molecule (28), and it will be interesting to define its cellular target functions in newly infected B lineage cells. The advantage provided to an ancient EBV mutant that "acquired" a (processed) copy of human IL-10 is surely significant if the gene enhances the primary transforming capacity of the virus. In this sense, escape from T cell surveillance may indeed be of lesser relevance as suggested by the fact that >90% of the adult population carries the virus but few succumb to primary EBV$^+$ B cell lymphoma unless there is a serious deficiency of immunocompetence (12, 13).

Our observations of the sequential expression of v- and then hIL-10 are intriguing. The experiments with antisense oligonucleotides as described here could not address the functional role of hIL-10 in the transformation process, but studies are under way with long-acting, stable phosphothioate oligonucleotides (49). It will be important to determine if there is a direct link between vIL-10 and hIL-10 expression, perhaps analogous to the *bcl*-2 transactivation by the LMP-1 latency gene (47). Interestingly, there is an EBV homologue of cellular *bcl*-2 in the BHRF1 reading frame (50).

The effectiveness of short-lived antisense oligonucleotides demonstrates that early EBV gene expression events show critical timing. While the expression of EBNA and LMP transcripts is closely linked and begins very quickly after infection (18), the IL-10 effect is required several hours later and targeting of IL-10 does not directly affect expression of the earlier latency genes. One attractive model derived from these data is the initiation of growth transformation by a fine-tuned barrage of quasi-cellular proteins, expressed out of context of the normal regulation of cellular gene expression and growth control. It is perhaps the sequence and timing of such "signals" that avoid triggering of normal cell growth control mechanisms while still driving the cell towards the first of continuous cell cycles.

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