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Transcription Mapping and Expression Patterns of Genes in the Major Immediate-Early Region of Kaposi's Sarcoma-Associated Herpesvirus

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Viral immediate-early (IE) genes are the first class of viral genes expressed during primary infection or reactivation from latency. They usually encode regulatory proteins that play crucial roles in viral life cycle. In a previous study, four regions in the KSHV genome were found to be actively transcribed in the immediate-early stage of viral reactivation in primary effusion lymphoma cells. Three immediate-early transcripts were characterized in these regions, as follows: mRNAs for ORF50 (KIE-1), ORF-45 (KIE-2), and ORF K4.2 (KIE-3) (F. X. Zhu, T. Cusano, and Y. Yuan, 1999, J. Virol. 73, 5556–5567). In the present study, we further analyzed the expression of genes in these IE regions in BC-1 and BCBL-1 cells. One of the immediate-early regions (KIE-1) that encompasses ORF50 and other genes was intensively studied to establish a detailed transcription map and expression patterns of genes in this region. This study led to identification of several novel IE transcripts in this region. They include a 2.6-kb mRNA which encodes ORF48/ORF29b, a family of transcripts that are complementary to ORF50 mRNA and a novel K8 IE mRNA of 1.5 kb. Together with the IE mRNA for ORF50 which was identified previously, four immediate-early genes have been mapped to KIE-1 region. Therefore, we would designate KIE-1 the major immediate-early region of KSHV. In addition, we showed that transcription of K8 gene is controlled by two promoters, yielding two transcripts, an immediate-early mRNA of 1.5 kb and a delayed-early mRNA of 1.3 kb.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus-8 (HHV-8), is a newly identified gammaherpesvirus, belonging to *Rhadinovirus* genus (Chang *et al.,* 1994; Moore *et al.,* 1996a). Epidemiologic studies suggest that this virus is an etiologic agent of Kaposi's sarcoma (KS) (Ambroziak *et al.,* 1995). KSHV is also associated with primary effusion lymphoma (PEL) (Cesarman *et al.,* 1995) and multicentric Castleman's diseases (MCD) (Soulier *et al.,* 1995). KSHV has been shown able to transform human endothelial cells (ECs) efficiently. Infection of primary human endothelial cells by purified KSHV causes long-term proliferation and survival of these cells which are associated with the acquisition of telomerase activity and anchorage-independent growth (Flore *et al.,* 1998). Therefore, KSHV is a human tumor virus.

As a gammaherpesvirus, KSHV establishes latent infection in lymphoid cells. In latently infected cells that contain a limited number of the viral genomes, no infectious virus is produced. Only a limited number of viral genes are expressed during latency and referred to as latent genes. Five KSHV latent genes were identified, and they encode v-cyclin, latency-associated nuclear antigen (LANA), v-FLIP, kaposin, and vIRF-2 (LANA-2) (Dittmer *et al.,* 1998; Rainbow *et al.,* 1997; Kedes *et al.,* 1997; Muralidhar *et al.,* 1998; Sadler *et al.,* 1999; Burysek and Pitha, 2001). When latency is disrupted, KSHV can switch to a lytic life cycle (Renne *et al.,* 1996; Miller *et al.,* 1997). In the lytic phase, the virus expresses its lytic genes in a temporal and sequential order. A few viral genes are expressed independently of *de novo* protein synthesis and are classified as immediate-early genes. Early genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA replication. Late genes are expressed after viral DNA synthesis, and their expression is, in general, blocked in the presence of inhibition of viral DNA synthesis.

Several lines of evidence suggested that the lytic life cycle of KSHV appears to be important in KSHV pathogenicity. First, KS is an endothelial neoplasm and latent KSHV infection is established in lymphocytes well before the onset of KSHV (Moore *et al.,* 1996b). Therefore, reactivation of KSHV from a latently infected lymphoid reservoir seems a necessary antecedent step in KS development. Second, the viral load in peripheral blood mononuclear cells increases with progression to KS (Ambroziak *et al.,* 1995; Whitby *et al.,* 1995). Third, treatment of KS patients or AIDS patients at risk for KS with antiherpesviral drugs, such as Foscarnet and ganciclovir, that block lytic but not latent KSHV replication, results in regression of KS lesions or decease in the incidence of

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KS development (Morfeldt and Torssander, 1994; Martin *et al.,* 1999). The last, several cytokine-like proteins and potential transforming proteins are encoded in the KSHV genome and have been proposed to play important roles in KS development. Most of them are expressed mainly after the virus switches to a lytic cycle in PEL cells. Taken together, these observations suggest that reactivation of latent KSHV to lytic life cycle is an event not only important for viral propagation, but also crucial for viral pathogenicity.

Reactivation of herpesviruses is initiated and controlled by a small number of regulatory proteins encoded by viral immediate-early (IE) genes. IE genes are the first class of the viral genes expressed during primary infection or reactivation. As their transcription does not require prior viral protein synthesis, this class of genes is experimentally defined by their transcription following primary infection or reactivation in the presence of inhibition of protein synthesis. We have been interested in KSHV reactivation and working on identification and characterization of KSHV immediate-early genes. In a previous study, we selectively induced the transcription of KSHV immediate-early genes by stimulating BC-1 cells with sodium butyrate in the presence of cycloheximide. Then the induced KSHV mRNA sequences were isolated using a gene expression screen method. This study led to identification of several immediate-early transcripts originating from four regions of the viral genome in PEL cells. These regions were designated as KIE-1, -2, -3, and -4 (Zhu *et al.,* 1999). In KIE-1 region, a 3.6-kb mRNA was characterized through isolating and sequencing its full-length cDNA and found to encode three putative open reading frames, namely ORF50, K8, and K8.2. A 1.7-kb mRNA encoding ORF45 and a 2.0-kb mRNA encoding ORF K4.2 was identified in KIE-2 and -3, respectively. Among these IE transcripts, ORF50 encodes a transactivator (Rta) which is able to upregulate viral lytic genes and sufficient to disrupt viral latency in B cells (Sun *et al.,* 1998; Lukac *et al.,* 1998). ORF45 was recently found to inhibit virus-mediated interferon-α and -β induction by blocking the phosphorylation and nuclear translocation of cellular interferon regulatory factor-7 (IRF-7) (Zhu *et al.,* 2002).

In the present study, we further analyzed the expression of immediate-early genes of KSHV. A major IE region (KIE-1) that encompasses ORF50 and other genes was intensively studied to establish a detailed transcription map of this region. The structure and expression pattern of each IE transcript in this region was investigated. We identified two novel immediate-early transcripts, namely ORF48/29b and ORF50 antisense RNAs. We also showed that transcription of ORF K8 gene is controlled by two promoters, yielding two transcripts, an immediate-early mRNA of 1.5 kb and a delayed-early mRNA of 1.3 kb.

RESULTS

Transcription mapping of two immediate-early regions in the KSHV genome

Among the four regions in the KSHV genome that were found to be actively transcribed in the immediate-early stage of viral reactivation, immediate-early mRNAs were characterized in three of these regions. They are mRNAs for ORF50 (KIE-1), ORF45 (KIE-2), and ORF K4.2 (KIE-3) (Zhu *et al.,* 1999). However, mRNA(s) originating from the fourth IE locus (KIE-4) which is located approximately between nucleotides 49,000 and 50,000 in the viral genome was not characterized (nucleotide numbers are according to Russo *et al.,* 1996). In addition, Northern analyses showed that multiple transcripts are expressed from KIE-1 locus in both directions, but only ORF50 mRNAs of 3.6–3.8 were characterized in this region (Zhu *et al.,* 1999). To continue characterizing immediate-early transcripts of KSHV, we systematically surveyed gene transcription in KIE-1 and KIE-4 regions. A series of single-stranded cDNA probes were prepared to scan these two regions for transcripts in both orientations. Positions and orientations of these probes are shown in Fig. 1. These probes were used to probe Northern blots containing poly(A+) RNA prepared from BC-1 cells that had been induced with sodium butyrate for 4 and 20 h in the absence or presence of cycloheximide (50 μg/ml), as well as from BCBL-1 cells treated with TPA (20 ng/ml) for 4 and 20 h. Cycloheximide at 50 μg/ml was shown to block cell protein synthesis within 30 min as indicated by very low percentage ($<$5%) of [^{35}S]methionine incorporation by treated cells (Zhu *et al.,* 1999). Cycloheximide was added 4 h prior to induction. Northern analysis of these mRNAs detected several transcripts in these two regions in both BC-1 and BCBL-1 cells (Fig. 2). These transcripts were classified on the basis of their transcription kinetics and are listed in Table 1. In KIE-4 region, probe 1 detected a 2.6-kb leftward mRNA in sodium butyrate induced BC-1 cells and TPA-stimulated BCBL-1 cells. The transcription of this mRNA in BC-1 cells can be induced regardless of the presence of cycloheximide (50 μg/ml) (Fig. 2A). This mRNA cannot be detected in BCBL-1 cells in the presence of cycloheximide at 50 μg/ml because BCBL-1 cells are hypersensitive to the toxicity of cycloheximide as previously reported by us and others (Zhu *et al.,* 1999; Lukac *et al.,* 1999). In the KIE-1 region, besides the rightward ORF50 mRNAs, there were a number of leftward transcripts that were transcribed in the IE stage. They include a 2.6-kb leftward mRNA that encompasses ORF48 (Fig. 2B) and a family of transcripts (7.4, 3.0, and 1.2 kb) that are complementary to ORF50 mRNA (Fig. 2D). The presence of cycloheximide (50 μg/ml) had little effect on the transcription of the 2.6-kb mRNA. The 7.4-, 3.0-, and 1.2-kb transcripts can be detected, but in reduced levels, in BC-1 cells that were treated with cycloheximide.

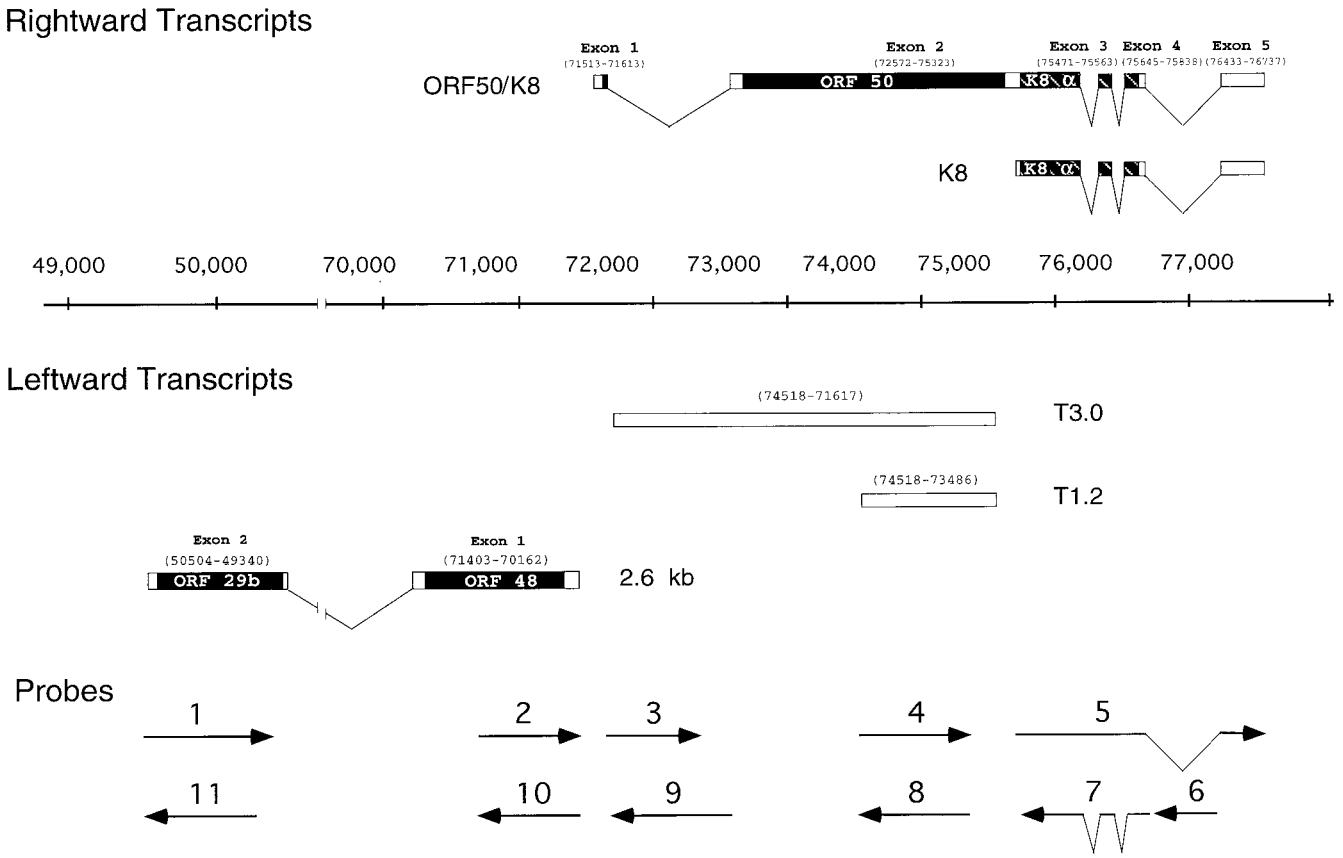


FIG. 1. Mapping of transcripts from the KSHV major immediate-early region. The upper portion of the figure illustrates the location, orientation, and splicing patterns of major transcripts from KIE-1 and KIE-4 regions. The lower portion shows the positions and orientations of single-stranded DNA probes that were used in the transcription mapping study.

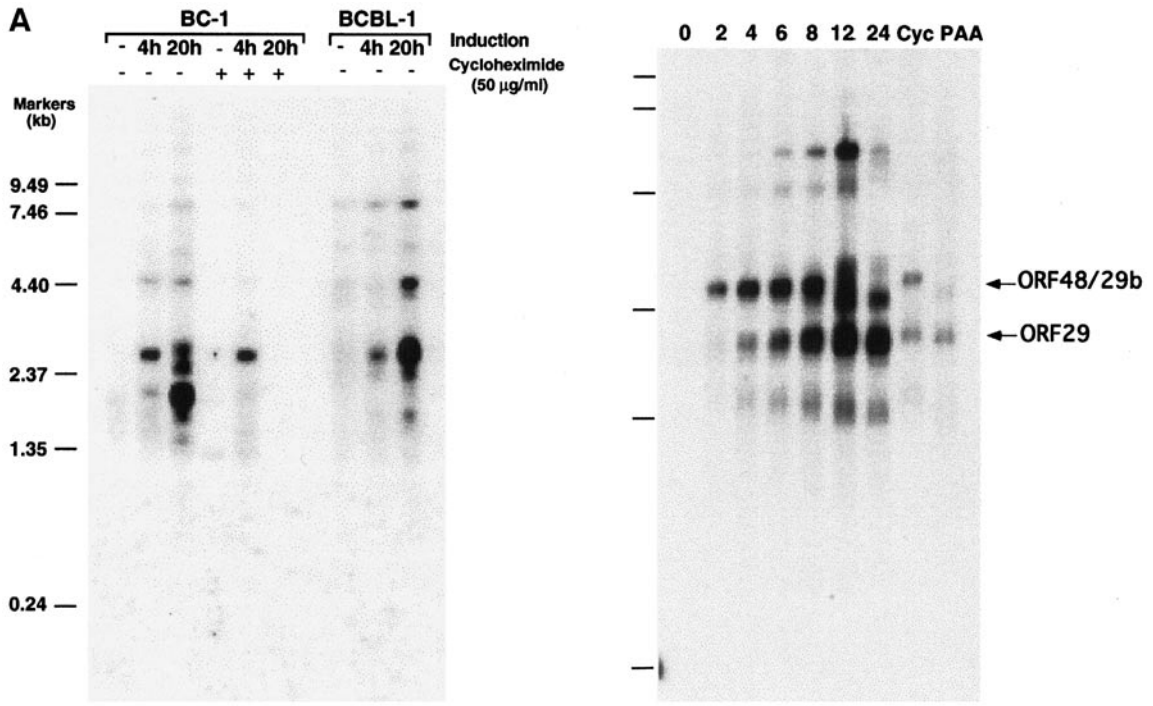
Probe 7 was designed to detect transcripts coding for ORF K8. Besides the immediate-early ORF50/K8 bicistronic mRNA (3.6 kb) and delayed-early K8 mRNA (1.3 kb) that had been reported previously (Lin *et al.,* 1999; Zhu *et al.,* 1999), a 1.5-kb transcript was detected in BC-1 cells at 4 h postinduction and the transcription of this RNA was not inhibited by the treatment of cycloheximide (Fig. 2F). Therefore, this mRNA is an immediate-early transcript. The transcription of two immediate-early mRNAs (3.6-kb ORF50/K8 and 1.5-kb K8 mRNAs) was found to be tightly controlled in the latent stage in BC-1 cells. In contrast, a considerable level of 1.3-kb K8 mRNA was detected in the latent stage (before induction) (Fig. 2F).

Several delayed-early and late transcripts were mapped in these two regions. A leftward ORF29 mRNA of 2.1 kb was detected by probe 1 but its transcription was inhibited in the presence of cycloheximide (Fig. 2A and Table 1). A leftward ORF49 mRNA of 1.1 kb was detected in TPA-induced BCBL-1, but not in sodium butyrate stimulated BC-1 cells (Fig. 2C). Probe 6 detected a 0.8-kb mRNA in BCBL-1 and BC-1 cells (Fig. 2E). This mRNA was described previously by others and known to encode a glycoprotein K8.1 (Chandran *et al.,* 1998; Raab *et al.,* 1998; Li *et al.,* 1999).

The kinetics of expression of the genes in these two regions were analyzed by determining the steady-state levels of these mRNAs in butyrate-induced BC-1 cells at intervals from 2 to 24 h postinduction. This experiment revealed that the ORF50 mRNA (3.6–3.8 kb), the 1.5-kb K8 mRNA, the 2.6-kb mRNA(s), and ORF50 antisense RNAs can be readily detected in 2 h of sodium butyrate treatment, while expression of the mRNAs for ORF29, ORF49, K8 (1.3 kb), and K8.1 were only detectable at or after 8 h postinduction (Fig. 2). The transcription of 1.3-kb K8 mRNAs was abolished in the presence of cycloheximide, but was not affected by the treatment with phosphonoacetic acid (PAA), a herpesviral DNA polymerase inhibitor. Thus, this mRNA is classified as a delayed-early transcript. The transcription of ORF29 and K8.1 were inhibited by both cycloheximide and PAA, classified as late transcripts.

Characterization of 2.6-kb IE transcript

Among the IE transcripts in these two regions, the 2.6-kb mRNA(s) and the transcripts complementary to ORF50 mRNA were not characterized. To characterize these IE transcripts, we constructed a cDNA library with



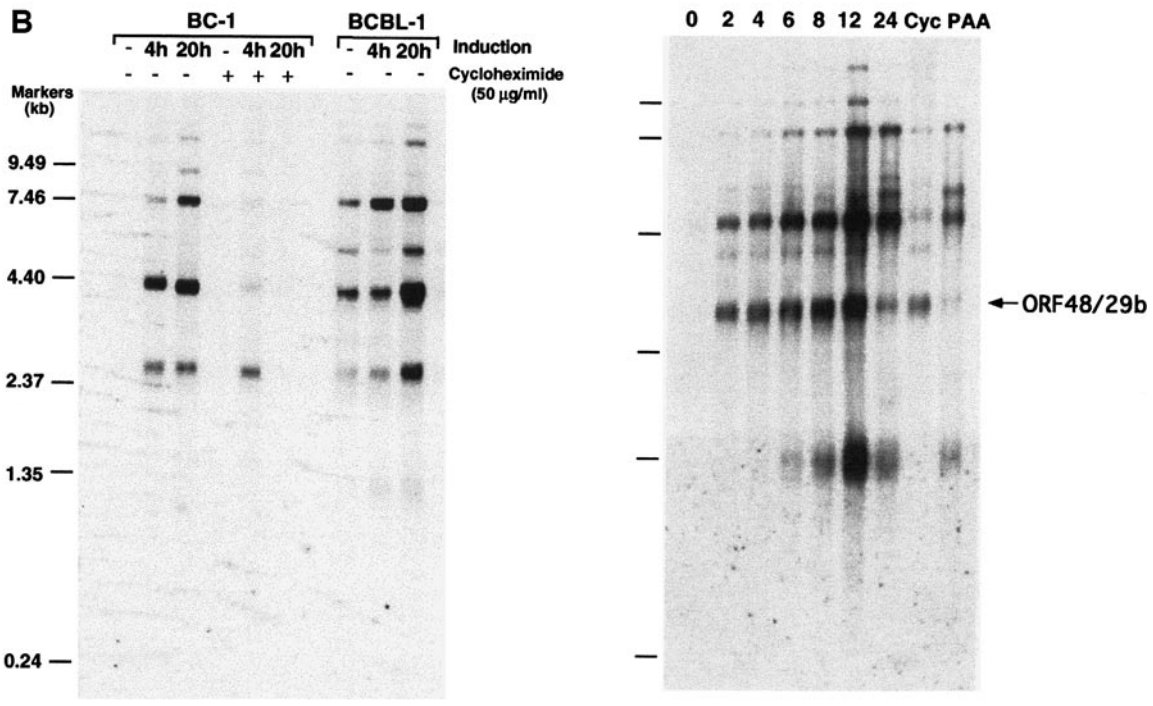


FIG. 2. Northern analysis of transcripts in KSHV major immediate-early region. Poly(A^+) RNAs were isolated from BC-1 cells that had been treated with sodium butyrate for 4 or 20 h in the absence or presence of cycloheximide and from BCBL-1 cells that had been induced with TPA as indicated above each lane. These RNAs were separated on a 1.0% agarose-formaldehyde gel and transferred onto nytran membranes. The membranes were probed with ^{32}P-labeled single-stranded DNA probes that were prepared using asymmetric PCR as described under Materials and Methods. The probes used for hybridization are indicated in the bottom of each panel, they are probes 1 (A), 2 (B), 3 (C), 4 (D), 6 (E), 7 (F), 8 (G) and 11 (H). The positions and orientations of these probes are shown in Fig. 1 and Table 1. The right panels are the kinetics of the expression of KSHV immediate-early mRNAs following an induction of viral reactivation in BC-1 cells. Cells were treated with sodium butyrate for 0, 2, 4, 6, 8, 12, 24, and 48 h. The mRNAs from cells that were induced with sodium butyrate for 4 h in the presence of cycloheximide (50 μg/ml) and cells that were induced for 20 h in the presence of PAA (0.4 mM) were also included. Molecular marker: 0.24- to 9.5-kb RNA ladder.

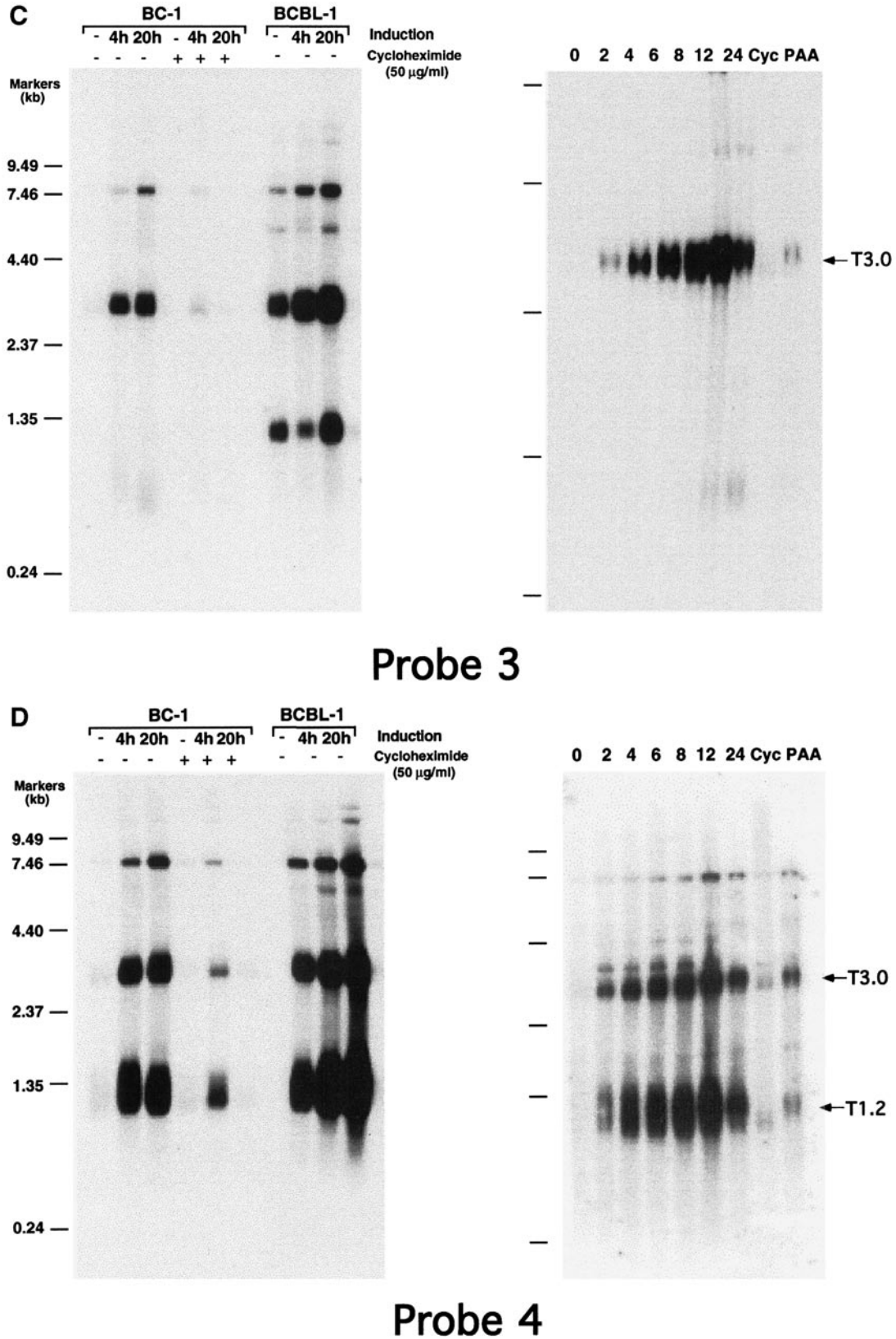


FIG. 2—*Continued*

mRNA prepared from BC-1 cells which had been stimulated with sodium butyrate for 4 h. This library was screened with two radioactively labeled cDNA fragments, probes 1 and 3 (see Fig. 1), aiming at obtaining cDNA clones for the IE mRNA originating from KIE-4 region and the transcripts complementary to ORF50

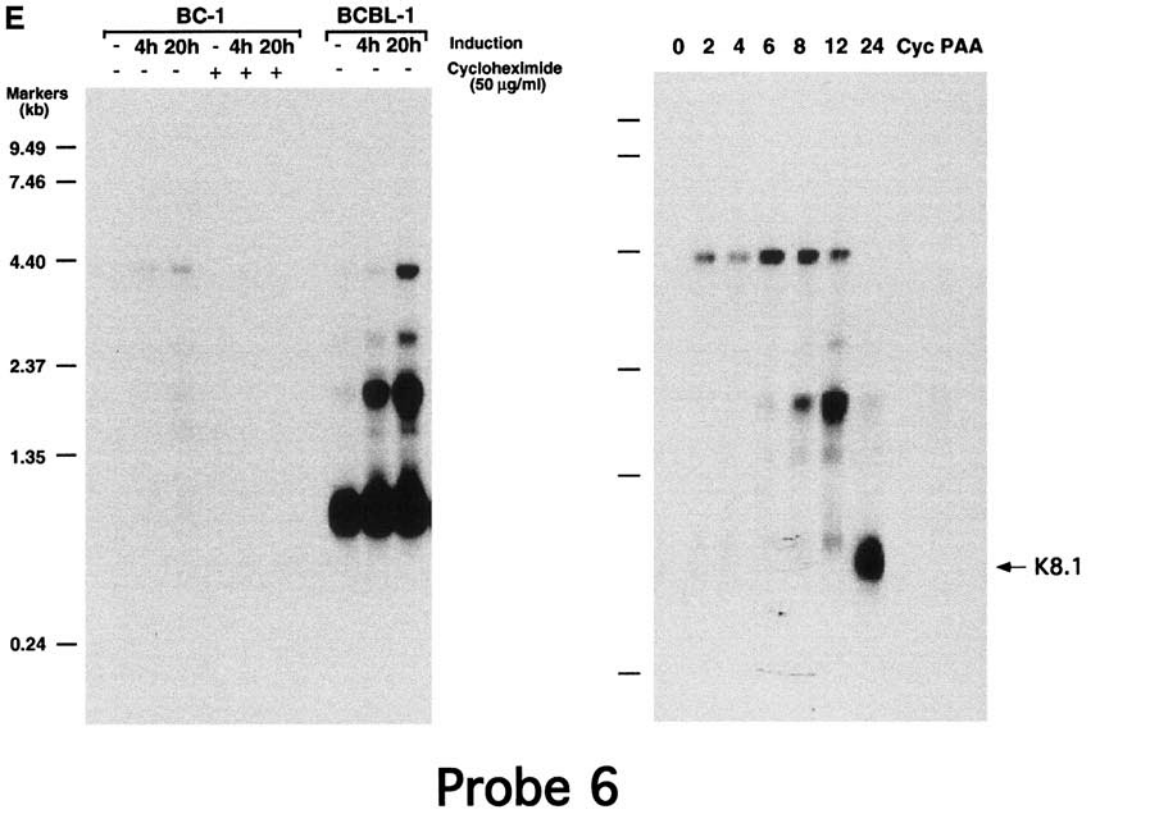
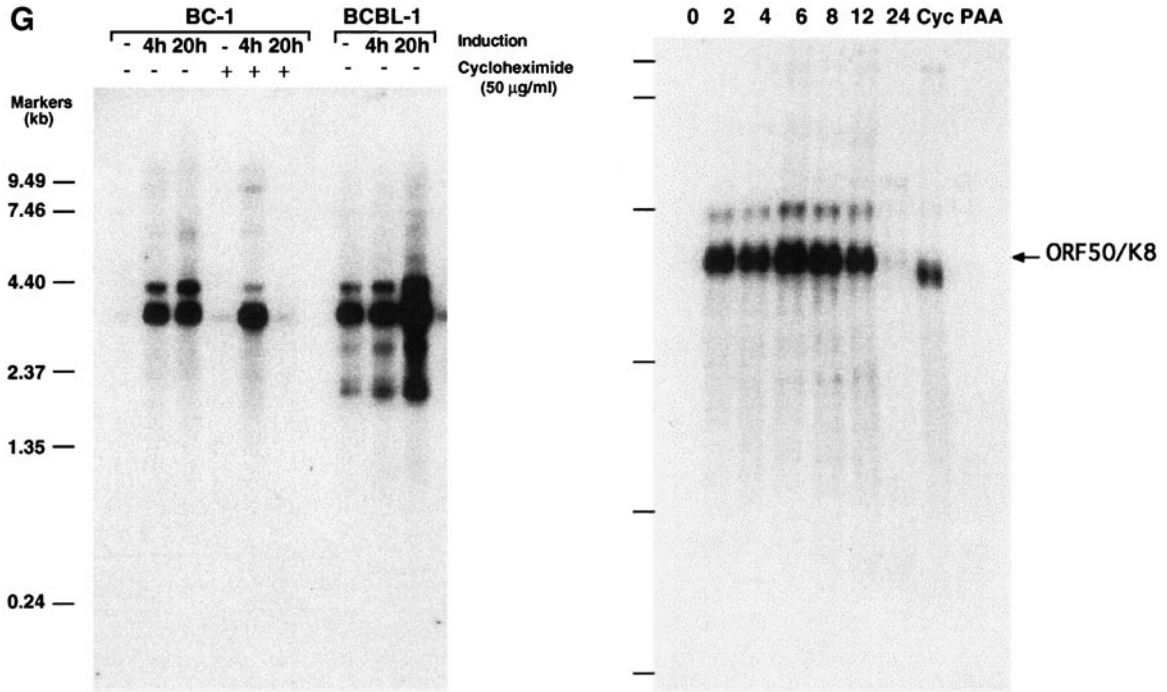


FIG. 2—*Continued*



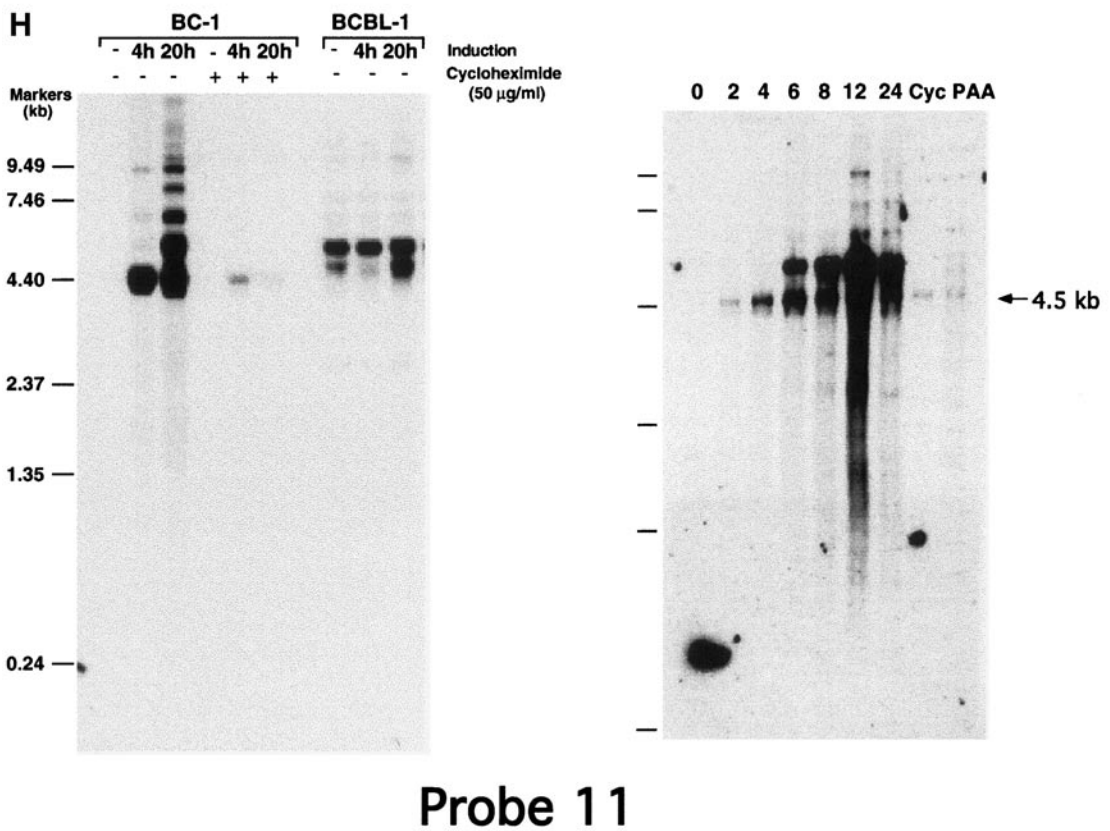


FIG. 2—*Continued*

mRNA. In the screen of the library with probe 1, two independent clones with inserts of 2.0 and 1.5 kb were isolated and sequenced. These two clones are terminated 20 and 17 nucleotides downstream of a consensus AAUAAA polyadenylation signal (nucleotides 49,366 to 49,361), respectively, followed by a poly(A) tail. The 5′

TABLE 1

Summary of Transcripts Detected in the Major Immediate-Early Region on the KSHV Genome

Probe no.	Position (bp) & orientation of probes	mRNA (kb) identified in butyrate-induced BC-1 cells	mRNA (kb) identified in TPA-induced BCBL-1 cells	Putative ORFs	Transcription in cycloheximide
1	49343–50398	2.6	2.6	ORF48, ORF29b	+
	(Rightward)	2.0	2.0	ORF29	−
2	70883–71402	7.4	7.4		+/−
	(Rightward)	4.4	4.4		+/−
		2.6	2.6	ORF48, ORF29b	+
3	71640–72298	7.4	7.4		+/−
	(Rightward)	3.0	3.0		+
			1.3	ORF49	
4	73486–74358	7.4	7.4		+/−
	(Rightward)	3.0	3.0		+
		1.2	1.2		+
5	74880–76737	ND	ND		
	(Rightward)				
6	76432–75839	4.3	4.3		
	(Leftward)	2.0	2.0		
		0.9	0.9	K8.1	−
7	75791–74880	4.3	4.3		−
	(Leftward)	3.6–3.8	3.6–3.8	ORF50, K8	+
		1.5	1.5	K8	+
		1.3	1.3	K8	−
8	74358–73486	4.3	4.3		−
	(Leftward)	3.6–3.8	3.6–3.8	ORF50, K8	+
9	72614–71640	ND	ND		
	(Leftward)				
10	71382–70883	ND	ND		
	(Leftward)				
11	50222–49343	5.0	5.0		−
	(Leftward)	4.5			+

ends of these clones are varied and located at nucleotides 70,947 and 70,516, respectively.

One of the cDNA clones was used to prepare single-stranded ^{32}P-labeled probe using asymmetric PCR for a Northern analysis of mRNA prepared from uninduced and induced BC-1 and BCBL-1 cells. The Northern analysis detected a 2.6-kb mRNA in both sodium butyrate induced BC-1 and TPA-treated BCBL-1 cells regardless of the presence of cycloheximide, confirming that the cDNAs we isolated were indeed for the 2.6-kb IE mRNA as detected with probes 1 and 2 in KIE-1 and KIE-4 regions (data not shown; the Northern pattern is similar to that in Fig. 2A).

Since the inserts of these two cDNA clones were only 2.0 and 1.5 kb in length, it appeared that none of them contains full-length cDNA of the 2.6-kb mRNA and the 5′ sequences of the mRNA were missing in both clones. To determine the 5′ end of the 2.6-kb messenger, a rapid analysis of cDNA ends (RACE) approach was used to obtain cDNA fragment containing the 5′ end of the mRNA. Two gene-specific primers (GSPs) were designed (T2.6RACE1: nt 50,022–50,048; T2.6RACE-2: nt 70,883–70,915) and used in the RACE assay. The 5′ RACE reaction generated a PCR product with an estimated size of 600 bp. The PCR products were cloned into pCR2.1 plasmid and four transformants were randomly picked for sequencing. The result revealed the major transcription initial sites at or near the nucleotides 71,402 and 71,403. The transcription initial site of the 2.6-kb mRNA is only 110 bp apart from that of the ORF50 mRNA that transcribes rightward. Thus, the IE promoter for 2.6-kb mRNA could overlap with the promoter controlling ORF50 gene.

Alignment of the 2.6-kb cDNAs with the KSHV genomic sequence (Russo *et al.,* 1996) revealed the presence of a large intervening sequence of nearly 20 kb (nucleotides 70,161 to 50,505) in the gene which specifies the 2.6-kb mRNA. The intervening sequence is framed by consensus donor and acceptor splicing signals and has been spliced out in the 2.6-kb mRNA (Fig. 3). The two exons of the 2.6-kb mRNA encode putative ORF48 and ORF29b, respectively (Fig. 3). Interestingly, the second exon was also found in the late ORF29 mRNA of 2.1 kb. A cDNA clone for the 2.1-kb mRNA was also isolated from a BC-1 cDNA library and was characterized. This mRNA consists of two exons and encodes an open reading frame

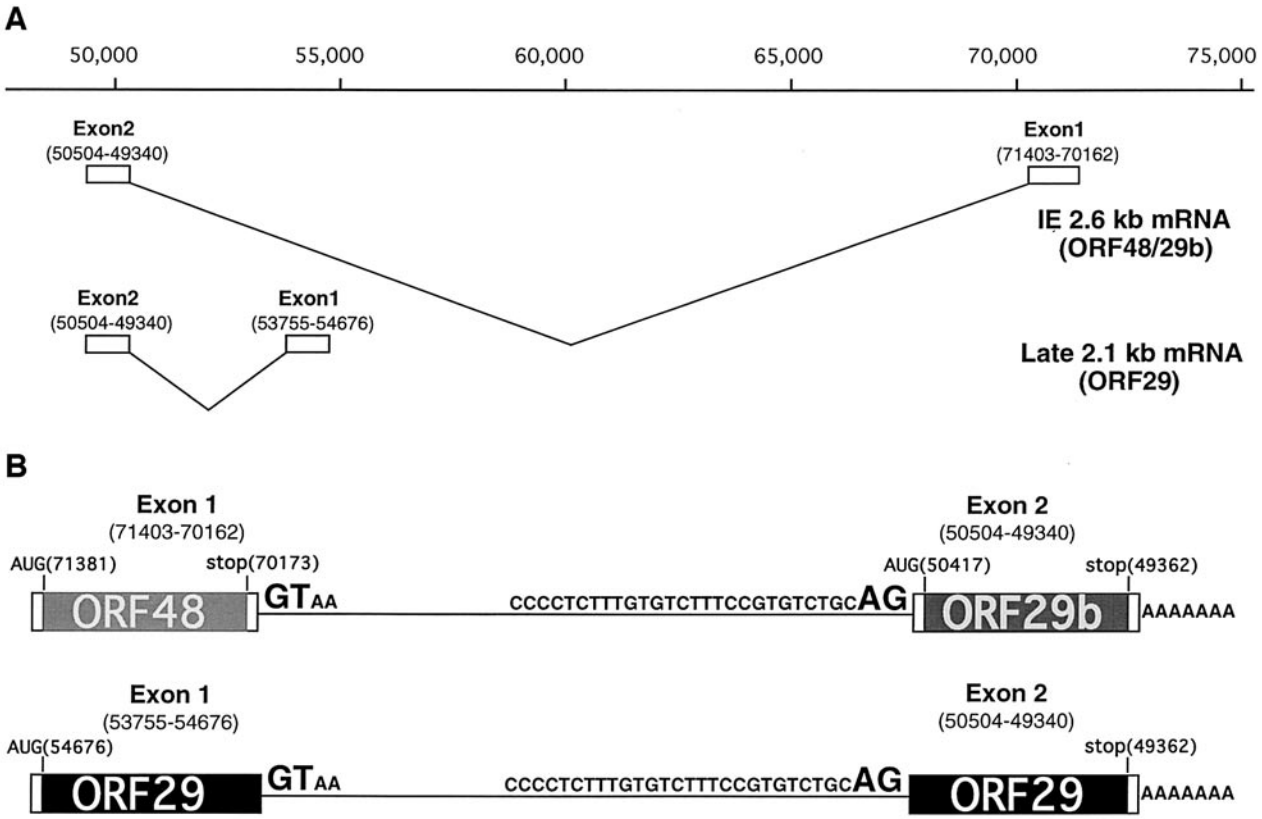


FIG. 3. Genomic organization of the genes for 2.6-kb immediate-early and 2.1-kb late ORF29 mRNAs (A). The splice donor and acceptor sites of the 2.6- and 2.1-kb mRNAs are compared in (B). The transcription start and stop codons for potential open reading frames in these mRNAs are indicated.

of 687 amino acids (ORF29). A comparison between the immediate-early 2.6 kb and late 2.1 kb mRNAs in their structures and splice sites is shown in Fig. 3.

Characterization of the transcripts that are complementary to ORF50 mRNA

Transcription mapping of KIE-1 region revealed three leftward transcripts of 7.4, 3.0, and 1.2 kb in both BC-1 and BCBL-1 cells (Fig. 2D). cDNA clones for the 3.0- and 1.2-kb mRNA were obtained. The cDNAs for the 3.0-kb mRNA were isolated from sodium butyrate induced BC-1 cDNA library with the probe 3 (nucleotides 71,640 to 72,298; see Fig. 1). The cDNA for the 1.2-kb mRNA were obtained by a PCR-based cDNA procedure followed by cloning into pCR2.1 vector. Both 3.0- and 1.2-kb mRNAs initiate at or near the nucleotide 74,518. The 1.2-kb mRNA terminates 12 to 32 nucleotides downstream of a consensus AAUAAA polyadenylation signal between nucleotides 73,522 and 73,517. The 3.0-kb mRNA terminates 11 to 22 nucleotides downstream of the AAUAAA signal between 71,634 and 71,629. Both 1.2- and 3.0-kb mRNAs appear to be unspliced transcripts. No large open reading frame was found in either 3.0- or 1.2-kb transcripts. Thus, it is possible that these ORF50 antisense RNAs do not encode any peptide but function in RNA level. These two transcripts were designated T3.0 and T1.2, respectively (Fig. 1).

Characterization of immediate-early and delayed-early transcripts of ORF K8

The 1.5-kb K8 mRNA is an immediate-early transcript, while 1.3-kb K8 mRNA is a delayed-early (DE) transcript. Since the transcription of these two RNAs are under different transcriptional regulation, we speculated that the 1.5- and 1.3-kb transcripts differ in their 5′ proximal sequences. To reveal the structural difference between 1.5-kb IE and 1.3-kb DE K8 mRNAs, the 5′ RACE was performed to obtain cDNA containing the 5′ proximate sequences of both mRNAs. Two K8-specific primers (K8C: nt 75,791–75,777; K8RACE-1: nt 75,136–75,110) were used in the RACE analysis. The 5′ RACE reaction generated two PCR products with estimated sizes of 330 and 580 bp (Fig. 4A). The PCR products were cloned into pCR2.1 vector and clones were randomly picked for sequencing. Compilation of these sequences revealed two major potential transcription start sites of K8 gene, one ranging between nucleotide 74,589 and 74,602, another at or near 74,845. This result suggested that there are at least two major transcription start sites for K8 mRNA,

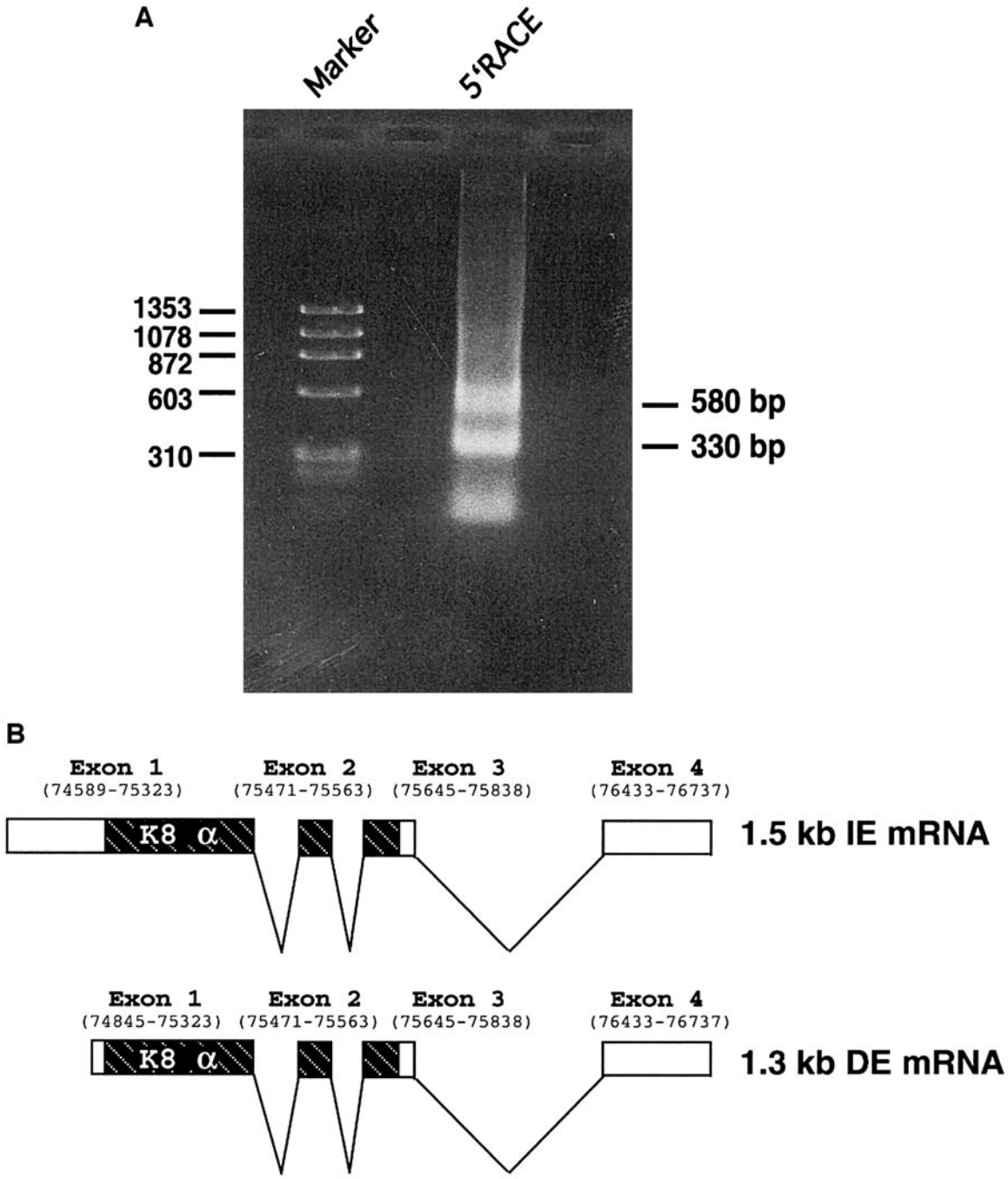


FIG. 4. (A) 5′RACE analysis of K8 mRNAs in BC-1 cells at 4 h postinduction with sodium butyrate. The ethidium bromide stained agarose gel shows two RACE products of 580 and 330 bp. (B) Schematic representation of structures of 1.5-kb immediate-early and 1.3-kb delayed-early K8 mRNAs.

presumably corresponding to 1.5-kb IE and 1.3-kb DE transcripts detected in Northern analysis (Fig. 4B).

The upstream sequences in front of these two transcription initiation sites have been analyzed for promoter activities. An IE promoter activity and a DE promoter activity were detected in their upstream sequences, respectively. The detailed information about the analyses of these two promoters will be published separately (Wang and Yuan, unpublished observations).

DISCUSSION

Viral immediate-early genes usually encode regulatory proteins that play essential roles in viral primary infection and reactivation from latency. Therefore, these genes always gain extraordinary attention in virological studies. In a previous study, we identified four loci in the KSHV genome that are actively transcribed in the immediate-early stage. Three immediate-early transcripts were

characterized in these regions: ORF50, ORF45, and K4.2 (Zhu *et al.,* 1999). In the present study, we continue to characterize KSHV transcripts in these regions. We carefully mapped two KSHV immediate-early regions, KIE-1 and KIE-4. This study led to identification of several novel immediate-early transcripts, including a 2.6-kb mRNA which encodes ORF48/ORF29b and a family of transcripts that are complementary to ORF50 mRNA. In addition, we found that K8 gene, which encodes a bZip protein, expresses two types of transcripts, 1.5-kb immediate-early and 1.3-kb delayed-early mRNAs. These two mRNAs are transcribed under control of two distinct promoters.

KIE-1 is a major immediate-early region

The original goal of this study was to characterize immediate-early transcripts in two immediate-early loci (KIE-1 and KIE-4) that are 20 kb apart in the KSHV genome. To our surprise, the 2.6-kb IE mRNA, which was originally detected in KIE-4 locus, is found to initiate from KIE-1 region and extend to KIE-4 locus. The transcription initiation site of 2.6-kb mRNA is only 110 bp apart from that of ORF50 transcript. In addition, a family of IE transcripts (T3.0 and T1.2) and a novel K8 IE mRNA of 1.5 kb were identified and mapped in KIE-1 region. Thus, four IE promoters have been found in KIE-1 region; they are for ORF50, ORF48/29b (2.6 kb), K8 (1.5 kb), and ORF50 antisense RNAs. Therefore, we would designate KIE-1 locus the major immediate-early region in the KSHV genome.

The 2.6-kb IE transcript

The gene for the 2.6-kb IE mRNA consists of two exons that are predicted to encode ORF48 and ORF29b, respectively (Fig. 3). The structure of this gene is interesting in two aspects. First, two exons are separated by a very large intron (20 kb) in the KSHV genome. Second, the gene shares the second exon with another viral gene, namely ORF29. ORF29 also consists of two exons which are separated by a 4-kb intron harboring two viral genes on the opposite strand. The ORF29 gene and its splice arrangement are highly conserved among the herpesviral family (including three subfamilies, α, β, and γ). KSHV ORF29 is predicted to encode a protein of 687 amino acids. The counterparts of the protein in other herpesviruses, i.e., UL15 of herpes simplex virus type 1 (HSV-1), are known to involve the cleavage and packaging of viral DNA into capsids (Baines *et al.,* 1994). The KSHV ORF29 mRNA (2.1 kb) and the 2.6 IE mRNA differ in their 5′ sequences and share a common 3′ sequence from the same exon 2. Unlike 2.1-kb ORF29 mRNA, the 2.6 IE transcript appears to be a bicistronic transcript encoding ORF48 and a N-terminally truncated ORF29 protein with predicted molecular weight of 32 kDa (designated ORF29b). Interestingly, the truncated protein was detected in HSV-1 (also 32 kDa, designated UL15.5) (Baines *et al.,* 1997; Yu *et al.,* 1997; Yu and Weller, 1998). The function of HSV UL15.5 is unknown. Whether ORF29b protein is synthesized from the 2.6-kb KSHV mRNA has not yet been examined. Further analysis of expression of ORF48 and ORF29b as well as their functions await the availability of specific antibodies against KSHV ORF48 and ORF29b. Generation of these antibodies is in progress.

A family of transcripts complementary to ORF50 mRNA

Northern analyses with single-stranded DNA probes detected multiple, divergent transcripts in the ORF50 locus. The structure of the rightward 3.6- to 3.8-kb mRNA for ORF50 was described previously (Sun *et al.,* 1998; Zhu *et al.,* 1999; Lukac *et al.,* 1999). In this article, we identified and characterized three novel transcripts of 7.4, 3.0, and 1.2 kb that are transcribed from the opposite strand of ORF50 DNA template. An analysis using the GCG Frame Program suggested that these transcripts do not encode any large open reading frame. Although the possibility that these transcripts encode very small peptide(s) could not be ruled out, it is possible that these transcripts function at the RNA level. This hypothesis can be clarified by determining whether these RNAs are associated with polysomes in cells through a sucrose gradient analysis. Nevertheless, T1.2 and T3.0 RNAs are of interest because they serve as antisense RNAs of ORF50 mRNA. In recent years, several sense–antisense transcript pairs have been reported in either viruses or cells. For example, the 8.3-kb latency associate transcripts (LATs) of HSV and its 2.0- and 1.5-kb spliced products are antisense to immediate-early messengers ICP-0 and γ34.5 and have been proposed to function as repressor of lytic replication of HSV (Stevens *et al.,* 1987; Spivack and Fraser, 1987). Unlike LATs, the T1.2 and T3.0 RNA and ORF50 mRNAs were found to be expressed at the same stage of the viral life cycle. It suggests that there may be a new mechanism underlying the sense–antisense RNA pair for gene regulation. An unique antisense mechanism in regulating the stability of the basic fibroblast growth factor (bFGF) mRNA has been reported in amphibians, birds, and mammals (Kimelman and Kirschner, 1989; Borja *et al.,* 1994; Knee *et al.,* 1994). A transcript is synthesized in the opposite direction of the bFGF mRNA and overlaps part of the coding sequence of bFGF. The antisense RNA causes modification of the bFGF mRNA during maturation of *Xenopus* oocyte, converting some adenine residues to inosine in the overlap region (Kimelman and Kirschner, 1989). It is possible that KSHV ORF50 gene is regulated in a similar positive fashion by its antisense RNAs.

ORF K8 gene is expressed in IE and DE phases under control of two distinct promoters

ORF K8 was previously identified on a delayed-early transcript of 1.3 kb (Lin *et al.,* 1999). The mRNA is highly spliced, which generates three splice variants of K8 as a result of alternative splicing. K8α is the major form, which codes for a 237 amino acid protein with a basic-leucine zipper domain near its C-terminus and an acidic domain near its N-terminus. Such a structure was found in many transcriptional regulators that compose a large bZip family. The two other variants, K8β and K8γ, encode proteins sharing the N-terminal portion with K8α, but lacking the C-terminal Zip domain (Lin *et al.,* 1999; Zhu *et al.,* 1999). K8 protein, especially the α form, displays limited similarity to the ZEBRA protein of Epstein–Barr virus (EBV) and the c-*Jun* proto-oncogene product (Lin *et al.,* 1999; Zhu *et al.,* 1999). The EBV ZEBRA protein is a transcriptional activator which activates the viral lytic gene expression cascade and initiates the switch of EBV from latency to lytic life cycle (Miller, 1990). The ZEBRA also binds to EBV lytic DNA replication origin (*Ori-Lyt*) and its binding is essential for viral lytic DNA replication (Fixman *et al.,* 1995). KSHV K8 is a fascinating protein. Its bZip structure and homology to EBV ZEBRA protein suggest that it is a regulatory protein and may involve either transcription or viral lytic replication. Its complicated and highly regulated splicing pattern suggests its importance to viral life cycles. Recently, we found that K8 bZip protein binds to a DNA sequence within the KSHV lytic DNA replication origin (Lin and Yuan, 2002). Wu *et al.* (2001) reported that K8 is incorporated into KSHV DNA replication compartments formed with six core KSHV replication proteins. However, the function of K8 and its role in KSHV replication have not been understood. In this study, we found that K8 is expressed in both immediate-early and delayed-early stages in that the transcription of K8 is controlled by two different promoters. This result suggested that K8 may have two distinct functions in the two stages similar to its EBV counterpart, the ZEBRA protein.

MATERIALS AND METHODS

Cell culture

BC-1 (Cesarman *et al.,* 1995) cells were purchased from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 15% fetal bovine serum (Gibco-BRL). BCBL-1 (Renne *et al.,* 1996) cells were obtained from the NIH AIDS Research and Reference Reagent Program and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cultures contained penicillin–streptomycin (50 units/ml) and fungizone (1.25 μg/ml amphotericin B and 1.25 μg/ml sodium desoxycholate).

Chemical induction

BC-1 cells were induced with 3 mM of sodium butyrate (Sigma, St. Louis, MO). BCBL-1 cells were treated with 20 ng/ml of phorbol-12-tetradecanoate-13-acetate (TPA). When induction was accompanied by inhibition of protein synthesis, cycloheximide (Sigma) was added in the culture to 50 μg/ml 4 h prior to the induction.

Northern blotting and hybridization

Total RNA was isolated from cells using Trizol reagent (Gibco-BRL) and poly(A$^+$) mRNA was purified using the PolyAtract mRNA isolation system (Promega, Madison, WI). The mRNA was separated by electrophoresis in 1% agarose/6% formaldehyde gel in 20 mM MOPS buffer, pH 7.0. Each lane was loaded with mRNA from 2×10^7 cells. The RNA was transferred to a Nytran membrane and hybridized with a single-stranded ^{32}P-labeled probe. Single-stranded DNA probes were prepared using asymmetric PCR with linearized plasmid templates and specific oligonucleotide primers, which were either a plasmid vector primer (i.e., KS or SK primers in pBluescript) or a primer specific to an insert sequence. The labeling reactions were performed in 15 μl of reaction solution (1× *Taq* polymerase buffer, 16.67 μM each dATP, dGTP, dTTP, 1.67 μM dCTP, 5 μl [α-^{32}P]dCTP (800Ci/mmol, 10 μCi/μl, Amersham), 100 ng DNA, 20 pmol primer, and 2.5 units *Taq* polymerase). The PCR initiated with a denaturing step of 2 min at 94°C, followed by 15 cycles of sequential steps of 1 min at 94°C, 1 min at 50°C, and 3 min at 74°C. Finally, the reaction was extended for 10 min at 74°C. RNA loading equivalence was controlled by probing with β-actin cDNA. A measure of 0.24–9.5 kb RNA ladder (Gibco-BRL) was included in each agarose–formaldehyde gel and detected in Northern blots by hybridization with labeled λ DNA.

Construction of cDNA library and cDNA isolation

Poly(A+) mRNA prepared from sodium butyrate induced BC-1 cells (4 h postinduction) was used in cDNA synthesis using oligo(dT) primer and the Universal Riboclone cDNA system (Promega). The double-stranded cDNAs were ligated to *Eco*RI adapters and inserted into λZAP-2 vector (Stratagene, La Jolla, CA). The ligation mixtures were packaged by using a Gigapack Gold packaging extract and used to infect *Escherichia coli* (XL1-Blue MRF′ strain) according to the manufacturer's instruction (Stratagene). 10^6 plaques were screened by nucleic acid hybridization with radiolabeled-specific cDNA probes. Positive phages were picked and purified by two more subsequent steps of screening. Individual λZAP-2 phages in positive clones were coinfected *E. coli* cells (SOLR strain) with ExAssist helper phage where the λZAP-2 phages were converted into plasmids (pBluescript SK). The cDNAs inserts were then analyzed by sequencing.

Rapid amplification of cDNA ends

A RACE-ready cDNA pool was generated using the Marathon cDNA amplification kit with Advantage cDNA polymerase mix (Clontech, Palo Alto, CA). Poly(A^+) RNA was isolated from BC-1 cells that had been treated with sodium butyrate for 4 h. Double-stranded cDNA pool was synthesized with AMV reverse transcriptase and cDNA synthesis primer (a modified lock-docking oligo(dT) primer, Clontech). The cDNAs were ligated with Marathon cDNA adapter (Clontech).

To obtain the 5′ RACE fragment of 2.6-kb transcript, the RACE-ready cDNA pool was amplified first with T2.6-RACE1 (5′ TCCAAGGCACTTCTGAACCTGCTGGGC 3′, 50,022–50,048) and adaptor primer (AP1, provided in the Marathon cDNA kit). The PCR products were then amplified with T2.6-RACE2 (5′ CACCAAGGAGAACCTGGCGTCTTGCAAAGTTGA 3′, 70,883–70,915) and nested adaptor primer (AP2) from the kit. Similarly, the 5′ portion of K8 mRNA was obtained through two PCR reactions, where K8C (5′ TCAACATGGTGGGAGTGGCGCGTCC 3′, 75,791–75,767) and AP1 were used in the first reaction and K8-RACE 1 (5′ TCTGCATTCAGTGAGCATGGCAGATGT 3′, 75,136–75,110) and AP2 used in the second. DNA fragments obtained in the RACE reactions were cloned into T/A-type PCR cloning vectors, pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced.

Nucleotide sequence accession number

The cDNA sequences of ORF48/29b and T3.0 have been deposited in GenBank and were assigned the Accession Nos. AF402654 and AF402655.

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