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Localization of Epstein–Barr virus-encoded small RNAs by *in situ* hybridization

(U1 RNA/7SL RNA/adenovirus-associated RNA/Burkitt lymphoma cell line/Epstein–Barr virus-transformed cells)

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ABSTRACT **Human B lymphocytes latently infected with Epstein–Barr virus (EBV) synthesize two low molecular weight RNAs designated EBER 1 and 2. Using an *in situ* hybridization technique we have localized EBER 1 and 2 within the nucleus of single EBV-harboring B lymphocytes from established and recently transformed cell lines. As controls, the locations of the small nuclear RNA, U1, and the small cytoplasmic RNA, 7SL, were examined in HeLa and EBV-harboring cells. Because of possible functional similarities between EBERs and the adenovirus-associated (VA) RNAs, VAI was also localized; it appeared to be in the nucleus and cytoplasm, implying that VAI may have a different role than that of the nuclear-localized EBERs.**

The Epstein–Barr virus (EBV) genome encodes two low molecular weight RNAs designated EBER 1 and 2, 166 and 172 nucleotides (nt), respectively (1–3). They are transcribed by RNA polymerase III and exist as ribonucleoproteins complexed with the host cell protein La (2, 4). In latently EBV-infected cells, the EBERs are found in high concentration (up to 10^7 copies per cell). In these cells only a small portion of the EBV genome is expressed and so far only seven gene products have been identified (5, 6): the nuclear proteins EBNA 1–4, the membrane protein LMP, and the two EBER RNAs. Because these products are expected to play important roles in host cell transformation, replication of DNA, and/or maintenance of latency, their precise functions are of great interest. Only two are currently known: EBNA 1 is involved in maintenance of the episomal viral DNA (7, 8), and LMP is implicated in cell transformation (9).

The function of the EBERs in viral proliferation is also not understood. It has been proposed that EBERs act similarly to the small virus-associated (VA) RNAs synthesized during adenovirus infection (10, 11). The two VA RNAs, like the EBERs, are transcribed by RNA polymerase III from adjacent genes of similar length and character. The VA RNAs, however, are synthesized at a very high rate only late after virus infection, during the viral replicative phase (12–14). By contrast, the EBERs are found in all EBV-carrying transformed cell lines (1, 3), and, if cell lines are induced with phorbol esters to enter the replicative phase, the amount of EBERs per cell does not change (15). The VA RNAs have been shown to prevent the inhibitory action of double-stranded RNA-dependent protein kinase on translation (16–19) and thereby counteract the effects of interferon produced by the host. These analyses have been greatly facilitated by the production of adenoviral genomes that are deleted in one or the other VA gene (13).

The inability to obtain mutants of the EBV genome necessitates the use of different types of approaches for elucidating the function of the EBER RNAs. Their location within EBV-transformed cells would be valuable information, narrowing possible functions. The majority of the EBER RNAs is found in the cytoplasmic fraction after aqueous fractionation of cells (unpublished results). However, leakage of small RNAs out of the nucleus is a problem: certain of these species [e.g., 7SK (20)] are found in the cytoplasm after aqueous cell fractionation but are localized in the nucleus upon nonaqueous cell fractionation; others, like tRNA precursors, which are bound by the La protein (21), are known to undergo processing in the nucleus before export to the cytoplasm (22).

We therefore decided to use *in situ* hybridization to determine the cellular location of the EBER RNAs. This technique avoids the disruptive and diluting conditions required for subcellular fractionation and allows direct visualization of the location of RNA molecules. We have adapted previously described *in situ* hybridization procedures (23–30) to determine the location of small RNAs within single tissue culture cells. We have probed several types of EBV-transformed B lymphocytes and HeLa cells with ^{3}H-labeled single-stranded RNAs corresponding to both strands of each EBER gene. For comparison, we chose probes for three other highly abundant small RNAs: U1, a nuclear RNA that functions in pre-mRNA splicing (31); 7SL, a cytoplasmic RNA functioning as an integral part of the signal recognition particle (32); and the VAI RNA. The results obtained demonstrate the general applicability of the procedures described.

MATERIALS AND METHODS

Cells, Media, and Virus Infections. All lymphocytes and suspension HeLa cell lines were maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. Monolayer HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Monolayer HeLa cells were infected with wild-type adenovirus type 2 titrated beforehand to determine the optimal amount for infection (gift from D. Solnick). Twenty hours after infection, the cells were harvested, fixed, and embedded. RNA from infected cells was isolated and electrophoresed in a 10% polyacrylamide/urea gel. VAI RNA was found in amounts greater than 5S rRNA in the infected cells. Purified human umbilical cord B lymphocytes were infected with wild-type B95-8 EBV, and the subsequently transformed cells were allowed to grow out for 40 days, whereupon they were harvested, fixed, and embedded.

Recombinant DNA. The genes for the various RNAs to be analyzed were cloned into SP6 vectors (33).

U1. The *Bgl* II/*Bam*HI fragment of pBRNSU1 containing an entire human U1 gene (165 nt) (gift from M. Mangin) was cloned into the *Bam*HI site of pSP65. After digestion with

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Abbreviations: EBV, Epstein–Barr virus; EBER, Epstein–Barr-encoded RNA; VA, virus-associated; nt, nucleotide(s).

*Hin*dIII, the pSP65/U1 plasmid was transcribed to obtain antisense RNA of 315 nt, of which 52% was U1 specific.

7SL "S." A pUC8 vector containing 81% of the unique S fragment (155 nt) of a human 7SL gene (gift from E. Ullu) was digested with *Eco*RI/*Hin*dIII and ligated with similarly digested pSP64 or pSP65 vectors. The subsequent vectors were digested with *Hin*dIII (antisense, pSP65/7SL S) or *Eco*RI (sense, pSP64/7SL S) and transcribed to obtain RNAs of 163 (77% 7SL specific) and 151 (83% 7SL specific) nt, respectively.

7SL. The pUC13 7SL5'-2 plasmid (34) (which contained the entire 300-nt human 7SL gene) was digested with *Bam*HI/*Eco*RI and ligated with similarly digested pSP64 or pSP65 plasmid DNA. The pSP64/7SL plasmid was then digested with *Eco*RI and transcribed to yield sense-strand RNA of 371 nt (81% 7SL specific). The pSP65/7SL plasmid was digested with *Hin*dIII and transcribed to yield antisense RNA of 380 nt (79% 7SL specific).

EBER 1. The pJJJ1 (4) vector was digested with *Mst* II/*Puv* II, and the fragment that contained 89% of the EBER 1 gene was treated with the Klenow fragment and ligated into pSP65 digested with *Sma* I. After determining the orientation, the pSP65/EBER 1 plasmids (both orientations) were digested with *Bam*HI and transcribed to obtain RNAs of 175 nt (83% EBER 1 specific).

EBER 2. The pJJJ2 (4) vector was digested with *Bam*HI/*Apa* I and the fragment that contained the entire EBER 2 gene was treated with S1 nuclease and ligated to the *Sma* I site of pSP65. After determining the orientation, the pSP65/*Eco*RI plasmids (both orientations) were digested with *Hin*dIII and transcribed to obtain RNAs of 437 nt (40% EBER 2 specific).

VAI. The p123 vector (gift from D. Bogenhagen, Stony Brook) carrying the VAI gene and the pSP65 vector were digested with *Pst* I/*Bst* I and ligated; the resulting vector was digested with *Xba* I and transcribed to produce an antisense probe of 133 nt (56% VAI specific) containing 47% of the VAI gene. To obtain a sense probe, the pSP65 and p123 vectors were digested with *Xba* I/*Hin*dIII and ligated; the resulting vector was transcribed after digestion with *Bal* I to obtain an RNA of 267 nt (60% VAI specific), which contained the entire VAI gene.

SP6 Transcription. Single-stranded RNA probes were transcribed using the SP6 polymerase transcription system (33). After transcription, the mixture was extracted with phenol and the RNA was precipitated with ethanol, dried, and resuspended in 10 μl of TE (10 mM Tris·HCl, pH 7.5/10 mM EDTA). The [^{3}H]RNA was assayed on nitrocellulose using a liquid scintillation counter. The labeled RNA probes were characterized on 10% polyacrylamide/urea gels. All probes were single bands of correct size with minor amounts (<10%) of breakdown products.

***In Situ* Hybridization.** Approximately 10^6 cells grown in suspension culture were pelleted into Beem capsules at 800 g for 3 min and washed with phosphate-buffered saline (PBS). Spinning cells at higher speeds caused deformities in the cell structure. The cell pellet was fixed in freshly prepared 1% glutaraldehyde in PBS for 30 min at 4°C, washed three times with PBS, and dehydrated through a series of increasing ethanol concentrations, ending with two changes of 100% ethanol for 10 min each.

The cells were then immersed in a series of solutions leading to embedding in diethylene glycol distearate (25). They were incubated in a 1:2 1-butanol/ethanol mixture and then a 2:1 mixture followed by four changes of 1-butanol alone for 15 min each. This series was followed by incubation in a 2:1 mixture of 1-butanol/diethylene glycol distearate and then a 1:2 mixture for 10 min each at 60°C, ending with three changes of diethylene glycol distearate alone for 1 hr each. The tubes, filled with liquid diethylene glycol distearate, were allowed to harden at 20°C, making sure that bubbles did not form. The diethylene glycol distearate blocks were cut using glass knives and a Sorvall Porter–Blum ultramicrotome. Sections (0.5 μm) were placed onto subbed, acid-washed glass slides with water (23) and allowed to air dry. To remove the diethylene glycol distearate, the slides were immersed in toluene for 20 min at room temperature. The sections were then hydrated by exposure to an ethanol series of decreasing ethanol concentrations.

For hybridization, [^{3}H]RNA probe (500,000 cpm in 1 μl) was mixed with 10 μl of a formamide solution [50% formamide, 0.6 M NaCl/60 mM sodium citrate, 0.02% (each) Ficoll, bovine serum albumin, and polyvinylpyrrolidone, 0.5 mg of tRNA per ml] and placed over each section; an acid-washed siliconized coverslip (22 mm) was added and sealed with rubber cement. The prepared slide was incubated 20 hr at 56°C in a moist chamber. After hybridization, the slide was submerged in 0.15 M NaCl/15 mM sodium citrate and the coverslips were carefully removed. Finally, the slides were washed with 15 mM NaCl/1.5 mM sodium citrate for 1 hr at 56°C and stored in water. Slides were dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with water and exposed in a dry, light-tight chamber at 4°C for 1–4 days. After exposure, slides were allowed to equilibrate to room temperature, developed in Kodak D19 (1:1 with water), and fixed in 30% sodium thiosulfate. After air drying, slides were stained with a methyl green/pyronin Y stain (35) for 30 sec, rinsed with water, and dried. The sections were photographed with a Zeiss photomicroscope III with a 63× objective using Kodachrome 64.

RESULTS

***In Situ* Hybridization Technique.** Localization of small RNAs within small lymphocytes by *in situ* hybridization necessitated adaption of published procedures to obtain an adequate signal and resolution between the nucleus and cytoplasm. Because of the numerous steps involved, we began by combining what appeared to be the best aspects of published studies and then made needed modifications (23–30).

We chose to section cells after pelleting instead of analyzing them intact for several reasons. First, B lymphocytes do not grow as monolayers. Second, if cells are smeared onto a glass slide and used in the *in situ* hybridization procedure, the preparations produce high backgrounds.

Because B lymphocytes have large nuclei and little cytoplasm, a fixation and embedding procedure that would retain excellent subcellular morphology was required. Initially, we tried fixing cells with paraformaldehyde and embedding in methacrylate (29), but cell integrity was destroyed. Intact cells were recovered after fixation with a 3:1 ethanol/acetic acid mixture, but results were not consistent; this fixative has been shown not to retain RNA well (24). Thus, glutaraldehyde, which has been shown to retain RNA (24), was finally adopted. As embedding materials, methacrylate and polystyrene were tried but with limited success. Good preservation of cell structure was ultimately obtained by using diethylene glycol distearate (25) after fixing with glutaraldehyde.

The hybridization probes were ^{3}H-labeled single-stranded RNAs synthesized from SP6 promoters, generally containing the full-length complement of the RNA to be assayed (33). The advantages of such probes for use in *in situ* hybridization have been documented (28), but importantly for our work they allowed the construction of sense-strand probes to ascertain the specificity of the technique. All probes were first tested by hybridization to gel-fractionated whole lysate RNA transferred to diazobenzyloxymethyl-paper. In each case, we observed that a high hybridization temperature

(>55°C) was necessary to reduce nonspecific background. Thus, 56°C was used for hybridization to cell sections.

Acceptable contrast staining of the sections was obtained with methyl green/pyronin Y (35) after initial problems with Giemsa and toluidine blue. Methyl green/pyronin Y stains the cytoplasm light pink, the nucleus light gray, and the nucleoli dark pink.

Location of U1 and 7SL. As controls for the *in situ* hybridization technique, we first examined two small RNAs (U1 and 7SL) whose functional location within cells is known: nucleus and cytoplasm, respectively.

The U1 DNA was cloned in both orientations; however, the transcription of the sense strand was so inefficient that only the antisense strand could be used as a probe. In Fig. 1 *A* and *J*, the U1 antisense RNA was hybridized to the EBV-containing Raji Burkitt lymphoma line and HeLa cell preparations, respectively. Hybridization in both cell lines appears exclusively nuclear, with little or no signal in the cytoplasm or in the nucleolus. This location is entirely consistent with the function of U1 RNA in the splicing of pre-mRNA (31).

Initially, the entire 7SL gene was cloned in both orientations. When the 7SL sense-strand probe was used to hybridize to either HeLa or Raji cells, a nuclear signal was sometimes seen (results not shown). The 7SL antisense strand probe also produced unexpected results: a nuclear and cytoplasmic signal in HeLa and Raji cells (results not shown). Because the 7SL RNA contains *Alu* sequences, these signals could be due to hybridization to non-7SL *Alu*-containing transcripts (36, 37). We therefore recloned the unique S fragment of 7SL and transcribed both strands. When these probes were hybridized with Raji cells, the sense strand gave only background (Fig. 1*B*), whereas the antisense probe gave a complex pattern that appeared initially both nuclear and cytoplasmic (Fig. 1*C*). However, after examining many cells, we concluded that the 7SL signal was predominantly cytoplasmic, clustered around the nucleus (although this may not be apparent from Fig. 1). Although the pattern for 7SL is clearly not as definitive as that for U1, the 7SL results are not inconsistent with the observation that the rough endoplasmic reticulum, which is the site of signal recognition particle action, is located predominantly around the nucleus in Burkitt lymphoma lines (38).

In the preparations analyzed, in particular Fig. 1 *A* and *C*, some cells are undergoing mitosis (arrows); dark purple chromosomes appear in place of the nucleus. In these cells the signals obtained with the U1 and 7SL antisense probes occupy the full interior of the cells exclusive of the chromosomes. The locations of 7SL and U1 were also determined for Bjab, an EBV-negative Burkitt lymphoma line, and a cell line recently transformed by EBV with similar results (data not shown). We conclude that nuclear and cytoplasmic RNAs can be examined by using the procedure developed here and that locations assigned by *in situ* hybridization correspond to the known functional location of the small RNAs.

Location of EBER 1 and 2. Probes used to determine the location of EBER 1 and 2 included the entire length of each RNA and corresponded to both strands. Controls performed with the sense-strand RNA of EBER 1 or 2 on Raji cells (Fig. 1 *D* and *G*) produced only background signals. Likewise, background signals were recorded using HeLa, Bjab, Bjab-B1 (an EBV-converted cell line), and a cell line recently transformed by EBV (data not shown). Controls for the EBER 1 and 2 antisense probes were HeLa and Bjab cells, again for which only a background signal was observed (data not shown).

When Raji cells, which do contain EBERs, were probed with the EBER 1 or 2 antisense probes, hybridization was clearly nuclear, exclusive of the nucleolus (Fig. 1 *E* and *H*). The grain pattern was similar to that found using the U1 probe (Fig. 1 *A* and *J*). Because of the longevity of the Raji cell line, it might be argued that EBERs play no function in the continued proliferation of this cell line. Therefore, human umbilical cord B lymphocytes recently transformed by EBV were also assayed. The newly transformed cells, which are irregular in shape and not as large as the Raji cells, also gave a nuclear signal with EBER 1 and 2 antisense probes (Fig. 1 *F* and *I*). In addition, an EBV-converted cell line, Bjab-B1, which expresses EBERs, showed a comparable nuclear location for EBER 1 and 2 (data not shown). In mitotic cells, the EBER 1 and 2 probes produced a uniform signal, except in the region where the chromosomes were located, as observed with U1 and 7SL probes (Fig. 1 *A*, *C*, and *J*).

Location of Adenovirus VAI. Since EBERs and VA RNAs have been suggested to function similarly, the location of the VA RNAs in adenovirus-infected HeLa cells was investigated for comparison. Probes to determine the location of VAI RNA only were selected because this RNA is made at higher levels and deletion analyses have indicated that VAI contributes more significantly to function than VAII (13).

HeLa cells infected with adenovirus were harvested at 20 hr after infection and embedded. The resultant sectioned cells showed many deformities, including large vacuoles. The embedding of infected cells was repeated with several modifications, but the same result was obtained even though uninfected cells embedded in parallel showed good preservation of cell structure.

Fig. 1*K* shows a control using the VAI sense-strand probe on adenovirus-infected HeLa cells; only background is seen. By contrast, in Fig. 1*L*, a variety of patterns was obtained by using the VAI antisense probe. Some cells contained little or no signal, whereas those that were positive had signal in either the cytoplasm or nucleus, or both. Such inconsistent results may be due to heterogeneity in the cell population: some cells may not have been infected, whereas others may have progressed to various points in the viral replicative cycle. It is not known precisely where VAI functions, only that it interferes with the phosphorylation of the protein synthesis eukaryotic initiation factor eIF-2α, which is located in the cytoplasm (16–19). Nonetheless, the *in situ* hybridization results do show a clear difference between the location of VAI and that of EBER 1 and 2. The different locations of VAI and EBERs imply different functions for the two sets of RNAs.

DISCUSSION

In situ hybridization has been widely used to determine the location of specific RNAs in tissues or organisms (e.g., ref. 27), but only occasionally within individual cells (24, 26, 30, 39, 40) and rarely for small nonpolyadenylylated RNAs (29). The modified *in situ* hybridization procedure developed here utilizes single-stranded RNA probes consisting generally of the full-length complement of the RNA to be assayed. Sense-strand probes were found not to hybridize, and antisense probes gave no signal with sectioned cells in which there was no complementary RNA. The technique gave definitive results confirming the known location of the U1 small nuclear RNA and exhibited a distinctively different pattern, not inconsistent with the cytoplasmic location for 7SL RNA. Thus, the nuclear location determined for EBER 1 and 2 seems likely to reflect the true *in vivo* situation.

We have observed that the intensity of the *in situ* hybridization signal does not necessarily reflect the amount of a particular small RNA. U1, for example, gave a much stronger signal than EBER 1 or 2, even though the numbers of EBER 1 and 2 are about 10-fold greater than U1 in Raji cells (unpublished results) and the specific activities of the probes were roughly equivalent. This may reflect the extent to which the probes have access to the small RNAs. During fixation/embedding, not only must cellular substructure be main-

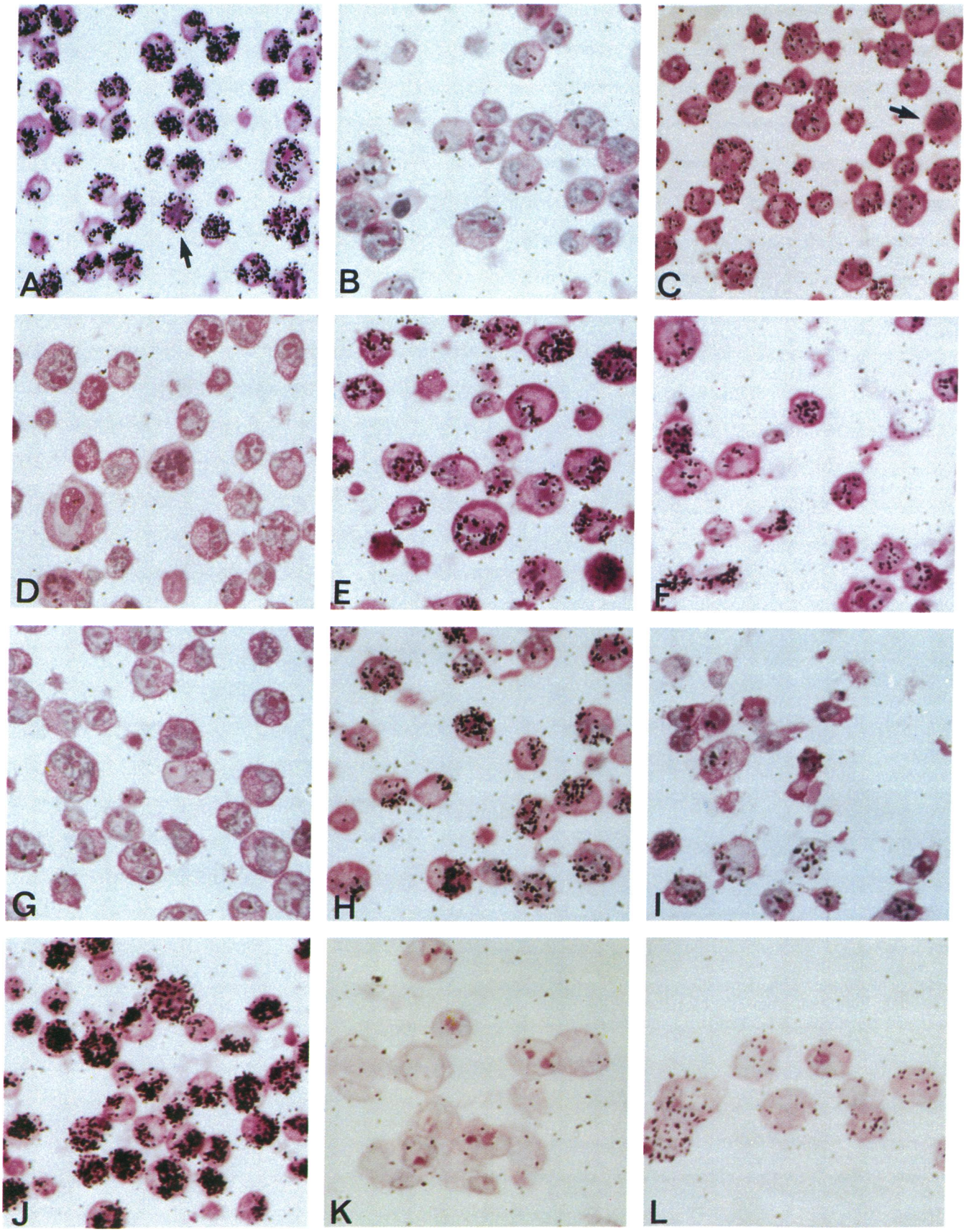

FIG. 1. Autoradiographs of cells hybridized *in situ* with ^{3}H-labeled single-stranded RNA probes to the small RNAs U1, 7SL, EBER 1, EBER 2, and VAI. Shown are methyl green/pyronin Y-stained cells (cytoplasm, light pink; nucleus, gray; nucleolus, dark pink). (×2000.) (*A–E, G,* and *H*) Raji cells. (*J*) HeLa cells. (*F* and *I*) Forty-day EBV-transformed human cord B lymphocytes. (*K* and *L*) Adenovirus-infected HeLa cells. (*A* and *J*) U1− probe. (*B*) 7SL S+ probe. (*C*) 7SL S− probe. (*D*) EBER 1+ probe. (*E* and *F*) EBER 1− probe. (*G*) EBER 2+ probe. (*H* and *I*) EBER 2− probe. (*K*) VAI+ probe. (*L*) VAI− probe. −, Antisense strand; +, sense strand.

tained, but the conditions must open up enough of an RNA's secondary structure so that it can base pair with an exogenous probe. Also, sufficient RNA must be retained by the sections to yield a convincing signal. Exactly how a particular small RNA assembles with proteins to form a specific ribonucleoprotein could clearly restrict access of regions of the molecule to a complementary probe. Likewise, association between the small ribonucleoprotein and other cellular structures could affect hybridization. U1 RNA therefore appears to be either better retained or more exposed to the exogenous probe than EBER 1 and 2, giving a higher signal. We conclude that the technique is limited in its ability to quantitate the level of one RNA relative to another but apparently reliable in its localization of small RNA species.

The nuclear location of EBER 1 and 2 is consistent with the predominantly nuclear location of the only protein known to bind EBERs, the La antigen (ref. 21; unpublished results). Greater than 95% of the EBER RNAs can be immunoprecipitated from EBER-containing cell lysates with autoantibodies directed against the La protein (unpublished results). The cytoplasmic location of EBERs observed after aqueous fractionation is likely an artifact resulting from leakage out of the nucleus, as has also been reported for the 7SK and U2 RNAs (20).

What can be inferred about the function of EBERs from their nuclear localization? Bhat and Thimmappaya (10, 11) have shown that EBERs can partially replace VAI function in cells infected with adenovirus deleted in this gene. We observed inconsistency in the location of VAI RNA from cell to cell, but the overall pattern suggested that VAI is located in the cytoplasm and nucleus. Because EBER 1 and 2 were consistently nuclear in location, their functions may well be different. We have tested the ability of EBERs to mimic the VAI RNA-induced inhibition of a double-stranded RNA-dependent protein kinase activity that phosphorylates a M_r 68,000 protein *in vitro* (19, 41). We found that EBERs had no inhibitory effect even when tested in the same extracts as VAI (results not shown). Other observations also suggest that the function of EBERs may not be identical to that of VAI. EBERs are transcribed in increasing amounts following EBV transformation of B lymphocytes (J.G.H., unpublished observations) but not following induction of viral replication by phorbol esters (15). Therefore, EBERs appear to act during latency, where they may be involved in transformation, DNA replication, or maintenance of latency. By contrast, there exist adenovirus-transformed cell lines that do not synthesize VA RNAs (42). Since EBERs are similar in structure to VA RNAs, and both mimic double-stranded RNA, it is still conceivable that EBERs act along the interferon pathway, in which expression of proteins is regulated by double-stranded RNA as a cofactor. Perhaps EBERs interfere with the anti-proliferative properties of interferon directly or indirectly, thereby preventing interferon from inhibiting the transformation and sustained growth of EBV-infected B lymphocytes.

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