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Influence of the Epstein-Barr Virus Nuclear Antigen EBNA 2 on the Growth Phenotype of Virus-Transformed B Cells

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Epstein-Barr virus (EBV) isolates show sequence divergence in the *Bam*HI YH region of the genome which encodes the nuclear antigen EBNA 2, a protein thought to be involved in the initiation of virus-induced B-cell transformation; type A isolates (such as B95-8 EBV) encode a 82- to 87-kilodalton EBNA 2A protein, whereas type B isolates (such as AG876 EBV) encode an antigenically distinct 75-kilodalton EBNA 2B protein. In the present work 12 type A isolates and 8 type B isolates have been compared for their ability to transform resting human B cells in vitro into permanent lymphoblastoid cell lines. Although the kinetics of initial focus formation was not markedly dependent upon the EBNA 2 type of the transforming virus, on subsequent passage type A virus-transformed cells (type A transformants) yielded cell lines much more readily than did type B transformants. Direct comparison between the two types of transformant revealed clear differences in several aspects of growth phenotype. Compared with type A transformants, cell lines established with type B virus isolates consistently displayed (i) an unusual growth pattern with poor survival of individual cells shed from lymphoblastoid clumps, (ii) a lower growth rate and a greater sensitivity to seeding at limiting dilutions, and (iii) a significantly lower saturation density that could not be corrected by supplementation of the medium with culture supernatant containing B-cell growth factors. This is the first direct evidence that, in EBV-transformed B-cell lines, the EBNA 2 protein plays a continuing role in determining the cellular growth phenotype.

The Epstein-Barr virus (EBV), an agent strongly associated with particular forms of B-cell lymphoma in humans (4, 22), readily infects human B cells in culture, transforming them into permanent cell lines in which every cell carries multiple episomal copies of the viral genome (14, 30; reviewed in reference 6). Only the so-called latent viral genes are constitutively expressed in transformed cells, hence the interest in identifying the full complement of these genes and in determining the contribution that each individual gene product makes to the transformation process. To date the best-characterized latent gene products are the nuclear antigens EBNA 1 encoded by the BKRFI reading frame of the viral genome (44, 45), EBNA 2 encoded by the BYRFI reading frame (8, 17), and EBNA 3 encoded by the BERFI reading frame (18) plus a latent membrane protein encoded by the BNLFI reading frame (16). Additional viral proteins may also be constitutively expressed in transformed cells (19, 41), but these have yet to be unequivocally identified.

Whereas EBNA 1 has been shown to play an important role in episomal maintenance of the viral genome in transformed cells (32, 33, 45), little is known directly about the function of any of the other latent gene products. There is circumstantial evidence, however, that suggests a role for EBNA 2 in initiating the transformation process. Thus the P_3HR_1 virus strain, in which a deletion has removed the entire EBNA 2-encoding sequence (2, 20, 31), differs from almost all other EBV isolates in having no in vitro transforming ability (25); indeed resting B cells do not even show a transient proliferative response to P_3HR_1 virus infection (11). Moreover in recombinants between P_3HR_1 virus and the resident viral genome in Raji cells, all of the transformation-competent viruses thus produced had acquired viral DNA fragments encompassing the EBNA 2-coding region (42).

Transfection experiments have recently been reported in which stable EBNA 2 expression has been achieved in a number of preimmortalized rodent cells (7, 28, 38, 46). Only one of these studies specifically addressed the question of a change in growth phenotype of the transfected cells and reported a slight relaxation of serum dependence in EBNA 2-positive Rat-1 cells (6). Whether this reflects some analogous role for the protein in determining the growth characteristics of EBV-transformed human B cells remains an open question.

In the present study we have exploited the unexpected discovery that natural EBV isolates possess one of two distinct EBNA 2 alleles. Type A viruses, of which B95-8 is the prototype, encode an 82- to 87-kilodalton EBNA 2A protein; type B viruses, of which AG876 is the prototype, encode a 75-kilodalton EBNA 2B protein (1, 5). The two products share some related sequence, particularly at the N and C termini, but their central domains show less than 50% amino acid conservation, and the polyproline domain, which is a feature of both proteins, is significantly shorter in EBNA 2B. Accordingly the proteins are antigenically distinct and induce antibody responses in humans which appear only weakly cross-reactive (5, 7, 38). None of the other known latent genes of EBV shows allelic variation of this kind, so that a comparison of a range of type A and type B virus isolates for their B-cell transforming ability could illuminate facets of the transformation process that are directly influenced by the EBNA 2 protein.

MATERIALS AND METHODS

Source of virus isolates. The B95-8 (24) and AG876 (29) cell lines were used as prototype sources of type A and type B virus isolates, respectively (5). A series of 18 EBV genome-

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positive Burkitt's lymphoma (BL) cell lines recently established from tumor biopsies and described fully elsewhere (23, 36, 37) were employed as a source of additional virus isolates. The IARC BL series was kindly provided by G. Lenoir, International Agency for Research on Cancer Laboratories, Lyon, France, and was largely derived from North African or French BL cases; the WW2 BL line was kindly provided by D. Moss, Queensland Institute of Medical Research, Brisbane, Australia, and was derived from a case of endemic BL arising in New Guinea; the remaining lines were developed in this laboratory from endemic African BL biopsies. For all of these lines, the EBNA 2 type of the resident virus had already been ascertained by hybridization of *Bam*HI-digested DNA fragments, after separation in agarose gels, with type A virus- and type B virus-specific probes (46; Young et al., submitted for publication).

Chemical induction of cell lines. The various EBV genome-positive cell lines, maintained in culture medium as described elsewhere (36), were exposed to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at a final concentration of 20 ng/ml for 3 days. At that stage the cells were harvested and washed free of TPA, and a small sample taken for the preparation of slides for VCA and EA staining. The VCA and EA status of the cells was determined by standard immunofluorescence testing with reference human sera as described elsewhere (13, 15) from counts upon 500 cells per slide. The remaining TPA-induced cells were X irradiated (4,000 rads) and then used as a source of virus in coculture.

Cocultivation assay for virus-induced transformation. Mononuclear cells were prepared from the blood of three adult seronegative donors as described elsewhere (26) and seeded at 2×10^6 cells per 2-ml well in 24-well plates with RMPI 1640 medium supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (FCS). To each well were added 2×10^5 X-irradiated, TPA-induced BL cells as a source of transforming virus; supplementation of the initial coculture medium with 0.1 μg of cyclosporin A per ml averted any nonspecific T-cell activation (34) and improved transformation efficiency. The cocultures were maintained by regular refeeding with culture medium (cyclosporin A free) over a period of at least 12 weeks and observed for the appearance of foci of transformed cells. Thereafter cocultures showing active growth were subcultured; as soon as possible the cells were transferred into upright 50-ml flasks, and the transformed cell lines were maintained thereafter by routine subculture.

EBNA 2 protein expression in transformed cells. Transformed cells were harvested from established cultures, solubilized in gel sample buffer (50 mM Tris buffer [pH 6.8], 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue), sonicated for 20 s, and then boiled for 5 min. Samples containing 10^6 cells in 20 μl were separated by the discontinuous gel electrophoresis technique of Laemmli (21) with a 5% acrylamide stacking gel and a 7.5% acrylamide resolving gel and blotted onto nitrocellulose filters (3). Excess protein-binding sites on the blotted filters were blocked by incubation for 2 h in 20 mM Tris-buffered saline, pH 7.5 (TBS), containing 5% dried skim milk (TBS-milk). The filters were then incubated at 4°C overnight with selected human sera, diluted 1:200 in TBS-milk, whose anti-EBNA 1 and anti-EBNA 2 status was known; the RWM serum was positive for anti-EBNA 2A (anti-EBNA $2A^+$), anti-EBNA 1^+, whereas the Am serum was anti-EBNA $2B^+$, anti-EBNA 1^-. The immunoblotted filters were washed in TBS containing 0.1% Tween 20 before incubating for 2 h with ^{125}I-labeled protein A (Amersham) which was diluted to 0.1 μCi/ml in TBS-milk. After extensive washings in TBS–Tween 20, the filters were dried and exposed to photographic film for 5 days without an intensifying screen. Molecular weight determinations were made from protein standards (Sigma Chemical Co.), which had been prestained with Remazole dye by the method of Griffith (12), run on the same gel.

Growth phenotype of transformed cells. Transformed cells were harvested from established cultures, washed, and suspended at a known concentration in fresh medium. The cell growth rate was determined by seeding the cells at one of a range of doubling dilutions in 0.2-ml microtest plate wells (6×10^2 to 4×10^4 cells per well); after 3 days each well received 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol) for the final 6 h of culture, and isotope incorporation into DNA was determined by scintillation counting of the acid-insoluble fraction. All proliferation assays were conducted in medium containing 10% FCS. Saturation density was determined by seeding cells at 4×10^4 cells per well as above in medium supplemented with either 5, 10, or 20% FCS or 20% FCS plus 20% conditioned medium derived from actively growing cultures of the IARC-BL18 cell line. The latter was kindly provided by J. Gordon from a stock known to have potent B-cell growth factor activity in in vitro assays (9, 10). These different types of culture were fed with half-volume changes of the relevant fresh medium every 3 to 4 days over a 2-week period, by which time saturation densities had been achieved. These densities were then determined by cell counting.

RESULTS

Rescue of type A and type B virus isolates. Table 1 identifies the 12 type A virus-carrying lines and the 8 type B virus-carrying lines used in the course of this work. Within both groups of lines there was a considerable spread in the level of productive virus infection observed after TPA treatment; the fraction of EC-VCA-positive cells ranged from <1 to >10%.

In cocultivation experiments with adult seronegative donor B cells, transforming virus was rescued from all 12 type A lines and from all 8 type B lines. Table 1 shows for each line, the proportion of cocultures in which transformed foci appeared and the mean time to their appearance. The kinetics of focus formation generally correlated with the virus-producer (EA-VCA) status of the TPA-induced cells in the coculture and was not obviously influenced by the EBNA 2 type of the particular virus isolate.

Differences between type A virus-transformed cells (hereafter referred to as type A transformants) and type B transformants become apparent, however, soon after focus formation. These differences were noted by using each of three separate populations of adult seronegative B cells as targets for transformation in the cocultures. Whereas foci of type A transformants expanded rapidly and could be subcultured with ease, type B transformants grew much more slowly, particularly in the early stages, so that transfer from 2-ml wells into culture flasks was often not possible until several weeks after the foci had first appeared. For this reason, established cultures of type A transformants were cryopreserved in early passage and resuscitated only when the parallel series of type B transformants was available for analysis. All of the ensuing experimental work was performed simultaneously on the two sets of lines within the first 3 months of their establishment in flasks.

EBNA 2 protein expression in transformants. When protein extracts from the various transformants were run on gels and

TABLE 1. Virus producer status of EBNA 2 type A and type B BL cell lines after TPA induction

Cell line	Antigen activity[a]		Transformed foci[b]	
	EA	VCA	No./ total	Time (wk) of appear-ance
Type A				
IARC BL 60	(+)	(+)	4/9	8
IARC BL 36	+	+	7/9	6
KYU-BL	+/++	+/++	9/9	4
MAK-BL	++	++	9/9	5
OBA-BL	++	++	9/9	4
IARC BL 37	++	++	9/9	2
IARC BL 18	+++	++/+++	9/9	3
IARC BL 72	+++	++/+++	9/9	2
MWI-BL	+++	+++	9/9	2
IARC BL 59	+++	+++	9/9	2
IARC BL 74	+++	+++	9/9	2
B95-8 prototype	+++	+++	9/9	2
Type B				
ELI-BL	+	+	6/9	6
CHEP-BL	+	+	9/9	5
IARC BL 29	+	+	5/9	5
WAN-BL	++	++	3/9	6
WW2-BL	++	++	9/9	4
MUK-BL	+++	+++	9/9	2
IARC BL 16	+++	+++	9/9	2
AG876 prototype	+++	+++	9/9	2

[a] Percentage of antigen-positive cells: (+), trace; +, <1%; ++, 1 to 10%; +++, >10%.

[b] Fraction of cocultures in which transformed foci appeared and mean time to appearance of foci. The combined results are shown from experiments with three different sources of seronegative donor B cells set up in coculture with each virus-producing line.

immunoblotted with relevant antisera, the expression of either an EBNA 2A or an EBNA 2B protein correlated exactly with the known EBNA 2 type of the original transforming virus. Figure 1 shows the reactions of eight transformants with each of two antisera. The anti-EBNA2A$^+$, anti-EBNA1$^+$ serum RWM reacts with EBNA 1 in all eight cell lines but detects a higher-molecular-weight EBNA 2A band only in those four cell lines established with type A isolates (the reactivity of these same extracts with a standard anti-EBNA2$^-$, anti-EBNA1$^+$ serum confirmed the identity of the EBNA 1 bands as assigned in Fig. 1a; data not shown). Conversely, the anti-EBNA2B$^+$, anti-EBNA1$^-$ serum Am detects EBNA 2B only in those four cell lines established with type B isolates (Fig. 1B). Note that the resolution of EBNA2B into two adjacent bands (Fig. 1b) has also been observed in other studies (Mueller-Lantzsch, personal communication); the precise relationship between these two molecular species of EBNA 2B has yet to be resolved. As anticipated from the earlier work on the B95-8 and AG876 virus prototypes (5), the EBNA 2 proteins encoded by type B isolates were significantly smaller than those encoded by type A isolates; EBNA 2B also appeared to show much less variation in size between different isolates when compared with EBNA 2A.

Growth phenotype of transformants. Microscopic observation of the transformed cell lines revealed clear differences between type A and type B transformants in their pattern of growth. As illustrated by the representative photomicrographs shown in Fig. 2, all type A transformants formed clumps but produced a healthy carpet of viable single cells between the clumps, whereas the type B transformants showed much tighter clumping and much poorer survival of single cells shed onto the base of the culture well. These morphological differences between cultures of type A and of type B transformants were maintained almost without exception over the 3-month period of observation.

Cell proliferation assays in limiting dilution conditions

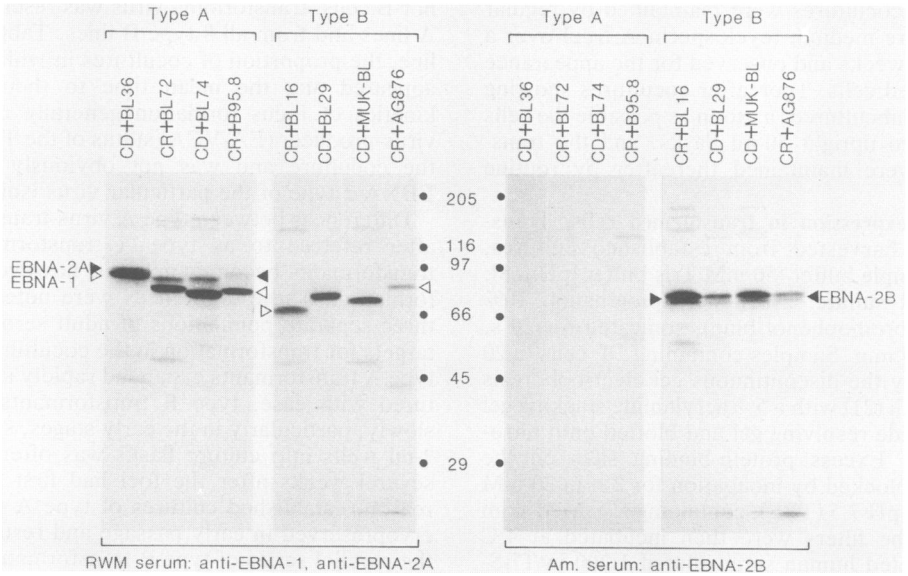

FIG. 1. Immunoblots showing the EBNA 2 protein expression in four representative type A and four representative type B transformants. The blots were probed with one of two human sera: (i) RWM, containing antibodies to EBNA 2A and EBNA 1, detected an EBNA 2A band (▶) and an EBNA 1 band (▷) in each of the type A transformants but only an EBNA 1 band (▷) in type B transformants; (ii) Am, containing antibodies to EBNA 2B but not to EBNA 1, did not react with any band in type A transformants but detected an EBNA 2B doublet band (▶) in type B transformants. The positions of the molecular size markers, given in kilodaltons, are shown (●).

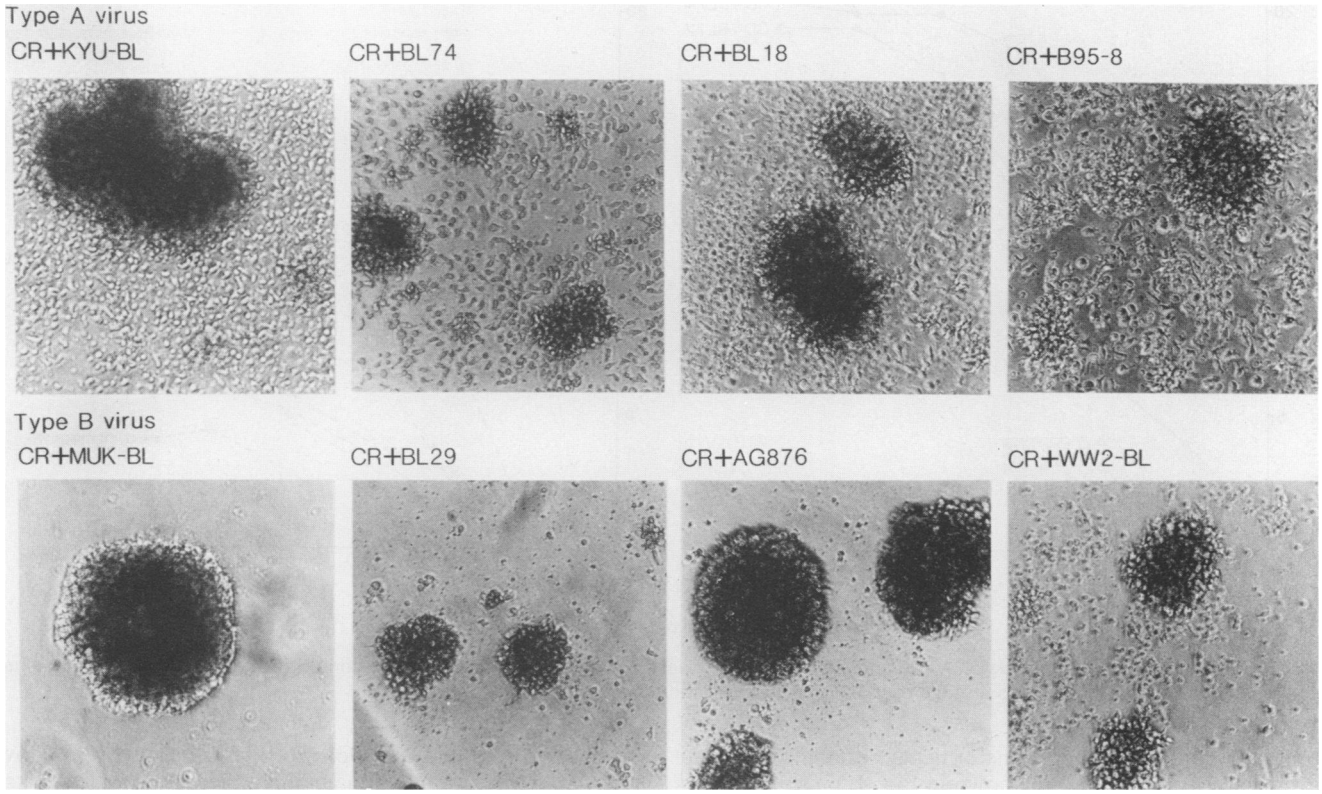

FIG. 2. Photomicrographs showing the typical appearance of type A transformants and of type B transformants growing in culture. Note that a carpet of viable lymphoblastoid cells, between the large clumps, is only seen with type A transformants.

confirmed the clear differences in growth rate between the two types of transformant. Figure 3 shows the results obtained with representatives of the panel of cell lines established from seronegative donor CR. In such experiments, cell seedings above 10^4 cells per microtest plate well produced optimal levels of proliferation (expressed as isotope uptake on day 3 per standard number of seeded cells), type A transformants grew significantly faster than type B transformants. At cell seedings below 10^4 cells per well, the growth rate falls increasingly short of optimal levels in both types of line, but the dilution effect appeared to be more marked in the type B transformants. Representative results

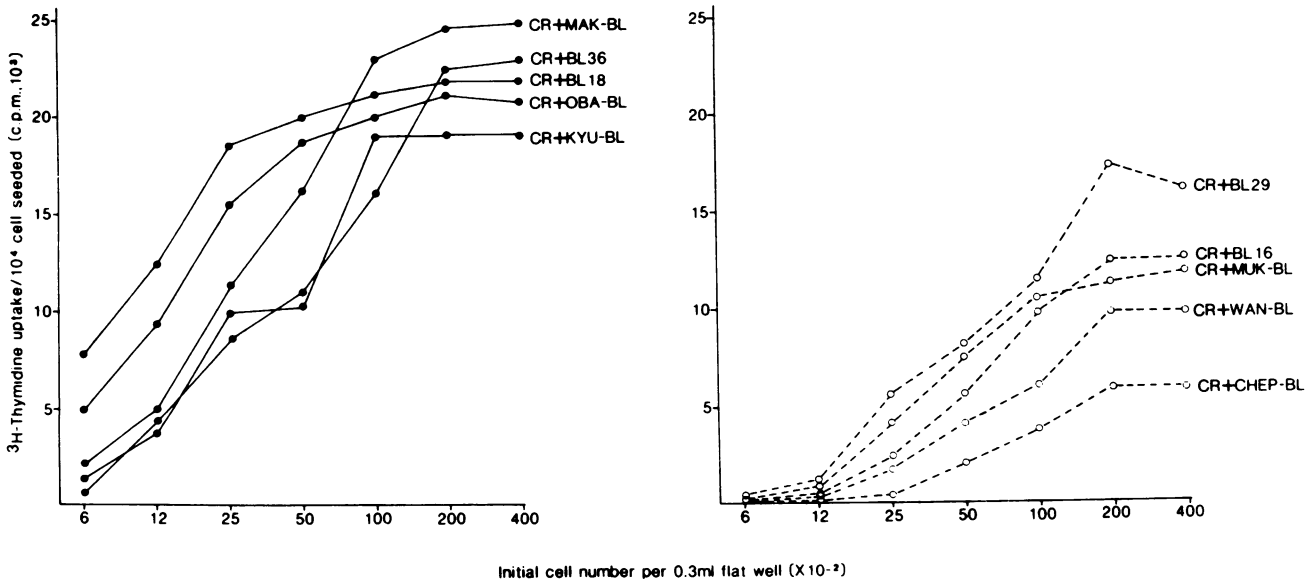

FIG. 3. Growth rates of five type A (●) and five type B (○) transformants, all established from donor CD, assessed by [^{3}H]thymidine uptake 3 days after seeding across a range of initial cell concentrations.

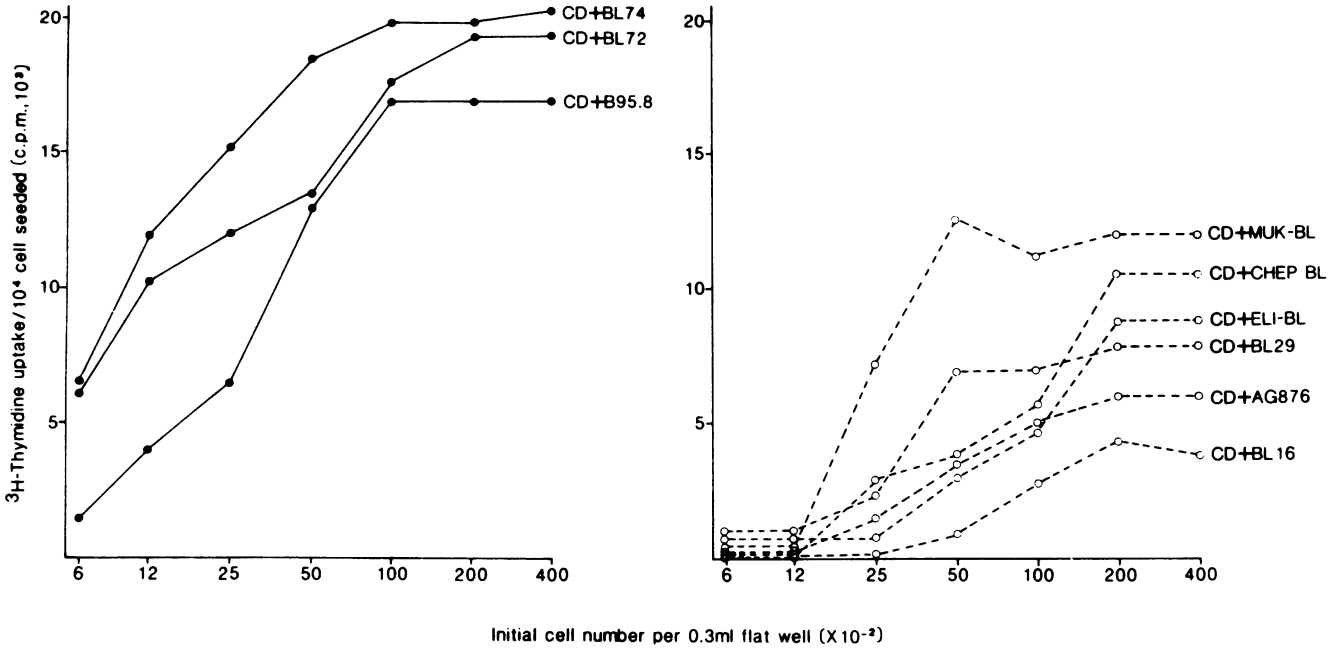

FIG. 4. Growth rates of three type A (●) and six type B (○) transformants, all established from donor CD, assessed by [^{3}H]thymidine uptake 3 days after seeding across a range of initial cell concentrations.

from a second series of transformants, established from seronegative donor CD, are shown in Fig. 4; once again the same differences in proliferation rate are apparent, as is the greater sensitivity of type B transformants to seeding at limiting cell dilutions.

In a final series of experiments, transformants were seeded into wells at a sufficient initial cell density to allow optimal proliferation and maintained thereafter for 2 weeks, with regular feeding, for the cultures to reach saturation density. The results both for donor CR and for donor CD transformants are shown in Fig. 5. At each of the three FCS concentrations tested, type A transformants achieved a

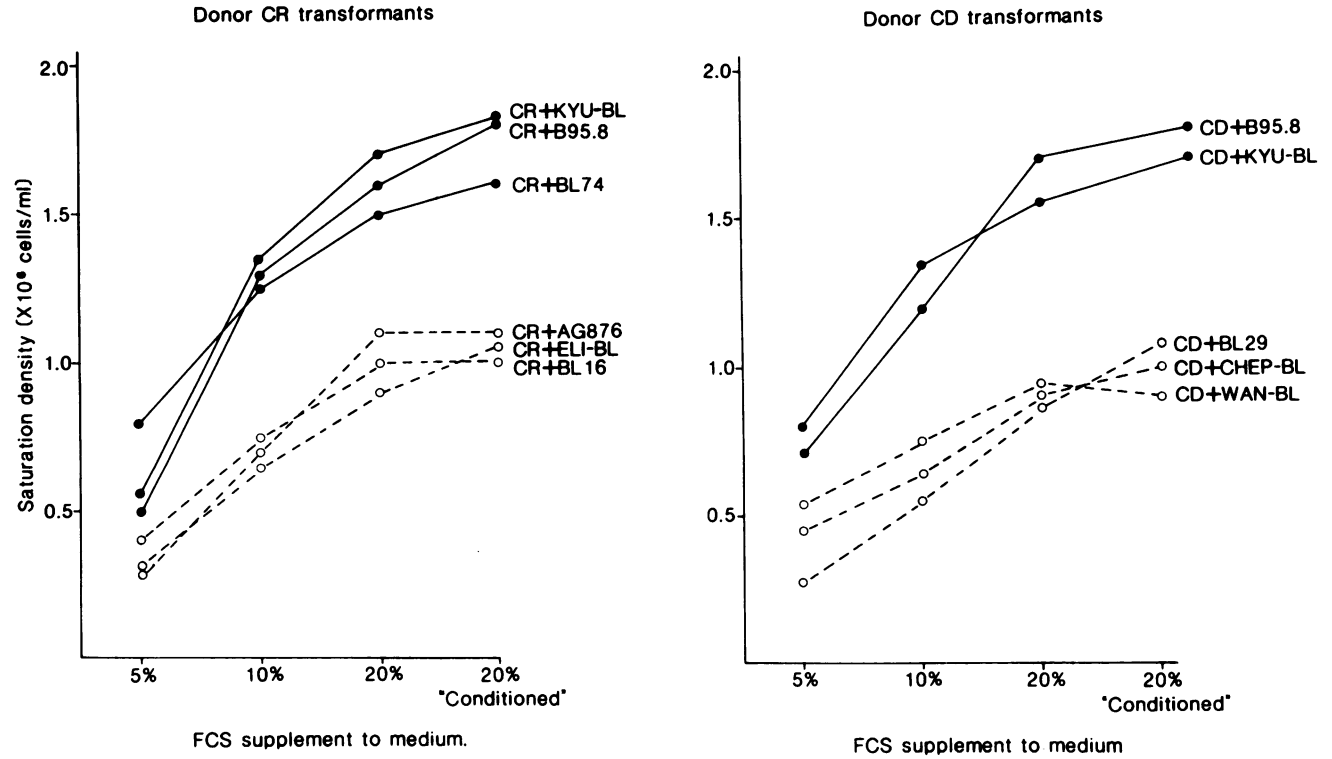

FIG. 5. Saturation densities of type A (●) and of type B (○) transformants, from donors CR and CD, in medium supplemented with different amounts of FCS plus conditioned medium containing B-cell growth factors.

significantly higher saturation density than did those of type B; these differences were still apparent even when medium containing 20% FCS was further conditioned with a known source of B-cell growth factors (in the form of culture supernatant from the BL18 cell line). Although Fig. 5 shows data on only a limited number of lines, these differences in saturation density between type A and type B transformants were regularly observed throughout the complete series of lines. In medium with 10% FCS, donor CD transformants achieved mean (± standard deviation) saturation densities of $1.25 \times 10^6 \pm 0.25 \times 10^6$ cells per ml for type A and of $0.7 \times 10^6 \pm 0.15 \times 10^6$ cells per ml for type B; under similar conditions donor CR transformants achieved mean values of $1.4 \times 10^6 \pm 0.2 \times 10^6$ cells per ml for type A and of $0.7 \times 10^6 \pm 0.4 \times 10^6$ cells per ml for type B.

DISCUSSION

The present experiments have exploited the existence of two families of EBV isolates, encoding structurally distinct EBNA 2 proteins (1, 5), to examine the possible functions of EBNA 2 in the process of virus-induced B-cell transformation. Since none of the other known EBV latent genes shows allelic variation of this kind, any consistent differences between EBNA 2A-encoding and EBNA 2B-encoding viruses in their transforming ability are very likely to involve aspects of the transformation process directly influenced by the EBNA 2 protein.

In the initial cocultivation experiments with a number of EBNA 2-typed BL cell lines as sources of transforming virus, there was no evidence of any differences in the kinetics of focus formation which might have been attributable to differences in the EBNA 2 type of the virus. Despite the semiquantitative nature of the results in Table 1, it can be seen that cocultures initially seeded with roughly equal numbers of type A virus-producing or of type B virus-producing cells went on to develop recognizable foci of lymphoblastoid growth after similar times. Furthermore, in experiments carried out before the present work, small resting B lymphocytes were exposed to cell-free preparations of B95-8 (type A) or of AG876 (type B) virus at doses already shown to induce equal levels of EBNA 1 expression in an EBV-negative target BL cell line. The kinetics of initial entry of these virus-infected B cells into S phase were essentially similar for the two virus types (unpublished observations). This does not argue against a role for EBNA 2 in the initiation of transformation; indeed the wealth of evidence from deletion mutants (2, 20, 31), from recombinant viruses (42), and from the kinetics of EBNA 2 expression postinfection (27) all argue for such a role. Rather the present experiments show that the EBNA 2A and 2B proteins appear to be functionally equivalent in this particular aspect of the transformation process.

Much more important are the clear differences in growth phenotype between type A and type B transformants which became apparent during cell line establishment and which in almost all cases persisted for at least the first 3 months of serial passage. Not only did type B transformants show an unusual pattern of growth (Fig. 2), quite distinct from the familiar appearance of in vitro-transformed lymphoblastoid cell lines established with type A viruses such as the prototype B95-8 strain, but also they displayed a significantly lower growth rate (Fig. 3 and 4) and achieved much lower saturation densities (Fig. 5) than did type A transformants. These differences cannot be explained by any obvious differences in EBV gene expression in the two types of transformant, since in this study (Fig. 1) and in the course of other work (39) we have shown that all the known transforming proteins, EBNA 1, EBNA 2 (A or B), EBNA 3, and latent membrane protein, are constitutively expressed in both types of cell line. It seems most likely, therefore, that the contrasting growth phenotypes noted here are a consequence of the presence within the cells of functionally distinct EBNA 2 proteins. Indeed these observations provide the first direct evidence of a continuing role for EBNA 2 in determining the growth phenotype of established EBV-transformed B cell lines.

In a wider setting, it is interesting to reflect that type B virus isolates, which are relatively common at least in equatorial regions of Africa and New Guinea although not in the West (Young et al., submitted), have completed effectively for survival alongside type A isolates in these regions despite their poorer B-cell transforming ability. Perhaps this transforming ability, as measured in vitro, is not critical for virus infectivity and persistence in vivo (35); alternatively, other factors present within these particular host communities may compensate for this apparent biological disadvantage of type B isolates. Certainly type B viruses are associated with a significant proportion of the cases of EBV-positive BL arising in the above equatorial regions (46; Young et al., submitted), as witnessed by the number of type B virus-carrying BL cell lines available for the present experiments (Table 1). Again, it may be that the performance of an EBV isolate in the B-cell transformation assay in vitro does not accurately reflect its potential for contributing toward B-cell lymphomagenesis in vivo. The two phenomena are different in several ways, not least in the identity of the infected target cell (36). Finally, it is interesting to note that the growth rate and saturation densities of the BL cell lines listed in Table 1, and serving as a source of transforming virus for the present experiments, were themselves independent of the EBNA 2 type of the resident viral genome (data not shown), in complete contrast to the results obtained with the derived in vitro transformants. This once again underscores our contention that when a malignant clone of EBV-positive BL arises in vivo, such a highly selected population is no longer dependent upon expression of the full set of viral transforming proteins for its continued growth (39).

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