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The Regulated Expression of Epstein–Barr Virus. III. Proteins Specified by EBV During the Lytic Cycle

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SUMMARY

The experiments show that 30 virus-induced or virus-specified proteins were synthesized in Raji cells after superinfection with Epstein–Barr virus (EBV) derived from P3HR1 cells. Using a combination of pulse labelling, application of cycloheximide blocks at different times post-infection, treatment with amino acid analogues and inhibition of DNA synthesis it was shown that three groups of proteins appear in Raji cells after superinfection; the synthesis of the proteins in any one group appears to be coordinately regulated. Amongst the six virus-induced proteins which were synthesized immediately after release from an early cycloheximide block one would expect to find those proteins essential for the transition from EBNA to EA synthesis. Using human sera with differing specificities for the various antigen groups 11 proteins were identified as being specifically precipitated by sera having high titres against the EBV-induced early antigen complex.

INTRODUCTION

Epstein–Barr virus (EBV) has a very restricted host range, being limited to B-lymphocytes of primates. Infection of these cells with EBV leads to immortalization of the cell rather than to a lytic infection (Diehl *et al.*, 1969; Gerber *et al.*, 1969) and the transformed cells carry the virus genome, express virus-determined antigens and in some cases spontaneously enter into a cycle of virus production (Miller *et al.*, 1972). Cells of the Raji line carry between 50 and 60 viral genomes, most of which are present as episomal-like structures rather than being integrated into the host genome (Tanaka & Nonoyama, 1974). The expression of the virus genome within these cells is limited to a single viral antigen: the EBV-determined nuclear antigen (EBNA). Treatment of these cells with iododeoxyuridine (IdUrd) (Glaser & Nonoyama, 1974), phorbol esters (zur Hausen *et al.*, 1978), butyric acid (Luka *et al.*, 1979), antibodies directed against human IgM (Tovey *et al.*, 1978) and antibodies directed against lymphocyte cell surface proteins (Bayliss & Wolf, 1979) leads to the synthesis of a second group of viral antigens – the early antigen (EA) complex which includes nuclear, cytoplasmic and membrane components. It has not been possible to induce a fully lytic cycle in Raji cells by the various treatments listed above. Superinfection of Raji cells, however, leads to a lytic cycle with release of progeny virus (Yajima & Nonoyama, 1976); up to 50% of the infected cells express virus capsid antigen (VCA) or EA (Bayliss & Nonoyama, 1978; Seigneurin *et al.*, 1977). It has been reported that at least nine virus-induced proteins could be identified in superinfected Raji (Raji SI) cells (Bayliss & Nonoyama, 1978) and that these proteins could be divided into two groups with respect to the dependence of their synthesis upon the replication of viral DNA. Further studies (Wolf & Bayliss, 1978) allowed the identification of three groups of coordinately synthesized proteins in superinfected Raji cells. In many ways these groups resembled the three coordinately synthesized groups of proteins defined by

Honess & Roizman (1974, 1975) in herpes simplex virus-infected cells. Our previous studies on proteins specific for EBV-infected cells (Bayliss & Nonoyama, 1978; Wolf & Bayliss, 1978) were restricted to observations made on nine virus-induced proteins and no correlation could be made between the biochemically defined groups of proteins and the immunologically defined antigen complexes. Since then immunoprecipitation studies on proteins extracted from EBV-producing cell lines, chemically induced cells and virus particles (Kallin *et al.*, 1979; Mueller-Lantzsch *et al.*, 1979; Thorley-Lawson, 1979) have shown that the EA complex contains at least 5 and perhaps as many as 16 proteins and that the VCA complex contains 2 major proteins (150 and 140×10^3 daltons) (Thorley-Lawson, 1979). The mol. wt. of these antigenically defined proteins resemble the mol. wt. described for a number of the previously identified proteins in Raji SI cells (Bayliss & Nonoyama, 1978; Wolf & Bayliss, 1978). In this study we report the identification of 30 proteins specific for Raji SI cells with mol. wt. between 150×10^3 and 29×10^3 , we classify them according to their regulatory properties and show that 11 proteins are specifically precipitated by antisera having high titres against the EBV EA complex.

METHODS

Tissue culture. P3HR1 cells were cultured in 32 oz prescription bottles at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum. When the cells reached a density of 10^6 cells/ml they were diluted 1:3 with fresh medium. Under these conditions the viability of the cultures remained high (over 95% by trypan blue exclusion). The cultures contained approx. 10% VCA-positive cells after maturation at 31 °C for several days. In order to increase the number of VCA-positive cells some cultures were treated with phorbol-12-myristate-13-acetate (40 ng/ml, procedure as described by zur Hausen *et al.*, 1978). Raji cells were cultured under similar conditions and were used as target cells for superinfection with EBV as described below.

EBV stock preparation. Cultures of aged P3HR1 cells were centrifuged at 4000 g for 15 min at 4 °C to pellet cells and cellular debris. The clarified supernatants were centrifuged at 27000 g for 2 h at 4 °C to pellet the virus. The resulting pellets were resuspended in a small volume (5 ml/l of original culture) of tissue culture medium and stored at -138 °C. Preliminary experiments indicated that whilst many virus stocks were able to induce EA synthesis in Raji cells they were unable to induce virus VCA synthesis. For this reason all virus stocks were pretested for their ability to induce the synthesis of late proteins as described previously (Bayliss & Nonoyama, 1978). Under ideal conditions each litre of culture supernatant yielded enough virus to superinfect between 2×10^7 and 4×10^7 Raji cells with production of VCA in up to 50% of the cells within 12 h after infection.

Radiolabelling of cells. Cells were collected by low-speed centrifugation and resuspended at a density of 2×10^6 cells/ml in methionine-free MEM culture medium supplemented with 2% heat-inactivated foetal bovine serum, containing between 10 and 50 $\mu\text{Ci/ml}$ [^{35}S]-methionine. The cells were returned to 37 °C. After the pulse the cells were washed with cold phosphate-buffered saline and were then analysed on SDS-polyacrylamide gels directly or following immunoprecipitation.

Immunoprecipitation. The washed, labelled cells were resuspended in cold IP buffer (1% Triton X-100, 0.1% SDS, 0.137 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 10% glycerol, 20 mM-tris-HCl pH 9, 0.01% NaN₃ and 1 $\mu\text{g/ml}$ phenylmethylsulphonyl fluoride) at a concentration of 10^6 cells/ml and disrupted by sonication (Branson, microtip at maximum output for 10 s), and incubated on ice for 30 min. The extracts were clarified by centrifugation at 180000 g for 30 min at 4 °C. In order to remove proteins which interact non-specifically with *Staphylococcus aureus* protein A absorbant the extracts were incubated with protein A coupled to Sepharose beads (Pharmacia) for 30 min at room temperature using 10 mg

beads/ml extract. The preabsorbed extract was mixed with 10 μ l EBV-positive or EBV-negative serum which had been preincubated for 30 min with an extract prepared from 10^7 unlabelled EBV-negative lymphoblastoid cells (BJA-B cells) at room temperature. Three mg of preswollen protein A beads were then added and after a further 30 min at room temperature the beads were washed with IP buffer until the washings contained no detectable radioactivity. The washed beads were resuspended in electrophoresis sample buffer and heated to 100 °C for 5 min to dissociate the immune complexes. The supernatant was analysed on polyacrylamide slab gels as described below. These procedures were adapted from methods described by Kessler (1975) and Thorley-Lawson (1979).

The following sera, obtained from the virus diagnostic laboratory of our institute, were used in the immunoprecipitation studies described in this paper: 6566-EBNA 1:32, EA:0; VCA 1:2048; 7070-EBNA 1:32; EA 1:4096; VCA 1:16384; 8463-EBNA 1:32; EA 0; VCA 1:1024. All three sera had membrane antigen (MA) titres higher than 1:320. In some experiments a pool of serum was used; this had the following titres: EBNA 1:400, EA 1:1200, VCA 1:6600, MA greater than 320. Negative control sera were also used; these contained no detectable antibodies against any of the EBV-determined antigens. The titres quoted above were obtained by indirect fluorescent antibody staining techniques described elsewhere: EBNA (Reedman & Klein, 1973), EA (Henle *et al.*, 1970), VCA (Henle & Henle, 1966) and for MA an adaption of the technique using ferritin-coupled anti-human IgG (Silvestre *et al.*, 1971) to the indirect fluorescent staining technique.

SDS-polyacrylamide slab gel electrophoresis (PAGE). Samples were dissolved in electrophoresis sample buffer (50 mM-tris-HCl pH 7; 2% SDS; 5% 2-mercaptoethanol; 30% sucrose) and heated to 100 °C for 5 min; with some samples it was necessary to reduce the viscosity by sonication as described above. The resolving gel contained 10% acrylamide, 0.26% diallyltartardiamide (DATD), 0.1% SDS and 0.375 M-tris-HCl pH 8.5, the gel length was 15 cm; the stacking gel contained 3% acrylamide, 0.08% DATD, 0.1% SDS and 0.12 M-tris-HCl pH 7. Polymerization was initiated by adding ammonium persulphate and TEMED. The upper and lower buffer tanks contained 0.025 M-tris, 0.192 M-glycine and 0.1% SDS (pH 8.5). Electrophoresis was carried out at room temperature using a constant current of 5.3 mA/cm² and continued until the leading front reached the end of the gel. The gels were fixed and stained for 1 h in a mixture of water, acetic acid and isopropanol (4:1:1) containing 0.1% Coomassie Brilliant Blue, destained in a mixture of water, acetic acid and isopropanol (8:1:1) and dried on to Whatman 3M filter paper under vacuum. Kodirex X-ray film or LKB 2208 Ultrafilm ³H were used to prepare autoradiograms of the dried gels. The apparent mol. wt. of the proteins were estimated by comparison with a set of standard proteins (*Escherichia coli* RNA polymerase 160×10^3 , 155×10^3 and 40×10^3 mol. wt. – a gift from Boehringer Mannheim – phosphorylase B 94×10^3 mol. wt., bovine serum albumin 68×10^3 mol. wt. and carbonic anhydrase 30×10^3 mol. wt.); approx. 200 ng of each polypeptide were applied per slot.

Metabolic inhibitors. Cycloheximide (CH) is an inhibitor of protein synthesis by preventing the translation of messenger RNA but not transcription. If added to virus-infected cells before the initiation of viral mRNA translation only those transcripts will be synthesized which can be made using the host transcription system; such proteins may be called alpha (Honest & Roizman, 1974), immediate early (Rakusanova *et al.*, 1971) or primary proteins. Addition of CH to infected cells after the initiation of primary but before secondary protein synthesis will permit the accumulation of mRNA for secondary proteins but not their synthesis. Similarly, in systems having tertiary protein synthesis addition of CH to cells after initiation of secondary protein synthesis will allow the accumulation of mRNA for the tertiary proteins. Removal of CH followed by a short pulse labelling will allow the identification of proteins translated from the pool of mRNA built up during the CH block. The drug was added to

cultures at a concentration of 50 $\mu\text{g/ml}$, and at the end of the treatment the inhibitor was washed from the cells with cold (4 °C) medium containing actinomycin D (2 $\mu\text{g/ml}$) to prevent further mRNA synthesis. The proteins synthesized immediately after removal of the CH block were labelled in the presence of actinomycin D. Canavanine and azetidine, analogues of the amino acids arginine and proline respectively, were added to the cells at 500 $\mu\text{g/ml}$. The cells were labelled in the presence of the analogues at the times indicated in the figure legends. Canavanine was used in arginine-free medium and azetidine in proline-free medium. DNA synthesis was inhibited with three different drugs: phosphonoacetic acid (PAA, 200 $\mu\text{g/ml}$), hydroxy-urea (HU, 4 mg/ml) or cytosine arabinoside (AraC, 50 $\mu\text{g/ml}$). The three inhibitors are reported to have different effects on the synthesis of host, endogenous viral and exogenous viral DNAs (Bayliss & Nonoyama, 1978; Mele *et al.*, 1974; Yajima & Nonoyama, 1976). The action of the three inhibitors is further described in the discussion.

Inhibitors were prepared as 100 times concentrates in Hanks' buffered saline, sterilized by passage through 0.22 μm filters and stored at -20 °C until required.

RESULTS

Time-ordered appearance of proteins synthesized in superinfected Raji cells

Actively growing Raji cells were harvested by low-speed centrifugation and mixed with an appropriate dilution of an EBV stock which had been pretested for its ability to induce late virus protein synthesis and inhibit host cell protein synthesis. Cells were shaken together with the virus for 1 h at room temperature, diluted to 2.5×10^5 cells/ml with MEM containing 2% foetal bovine serum, dispensed into culture tubes (10^6 cells/tube) and incubated at 37 °C in a CO₂ incubator. At various times after infection cultures were pulse-labelled (30 min) and prepared for SDS-PAGE. Fig. 1 illustrates the results of such an experiment. Mock-infected cells were prepared similarly excepting that the infection was done with virus-free culture medium. During the first 6 h after infection there appeared to be a slight stimulation of host cell protein synthesis; after this time the host proteins disappeared from the profiles and by 24 h post-infection very few host proteins can be identified. Previous experiments (Bayliss & Nonoyama, 1978) indicated that a single new protein could be identified in Raji SI cells shortly after the onset of infection; however, close inspection of the profiles presented in Fig. 1 reveals that six proteins (120, 110, 105, 102, 63, 54) were synthesized early after infection. Most of these proteins were made in small amounts and migrated close to host proteins, which may explain our previous failure to identify these proteins. The synthesis of these proteins increased until about 6 h post-infection and then began to decline. At this time a second group of proteins began to appear (150, 138/135, 90, 83, 80, 49, 45, 40, 35 and 33), their synthesis increasing steadily until about 12 h post-infection and then declining slightly. One major exception to this observation is protein 138/135, the synthesis of which was considerably reduced after 12 h post-infection; this band is a complex of two virus-induced proteins and one host protein. Band 90 is also a doublet, which was resolved in some gels but not in others. The last protein to appear in Raji SI cells is 143; its synthesis was initiated between 10 and 12 h post-infection and continued until 24 h post-infection when the experiment was terminated. The rather abrupt appearance of virus proteins at 8 h post-infection is partially artefactual since at this time host protein synthesis had considerably declined and in addition the amount of incorporated label dropped. It was therefore necessary to use two-stage exposures of the gel shown in Fig. 1, so that whilst the virus proteins are present in proportionally greater amounts the absolute amount present at 6 and 8 h may not drastically differ. When the tracks M 1, 2, 4 and 6 are exposed for the same length of time as the later tracks it becomes impossible to analyse the gel due to the ongoing host synthesis.

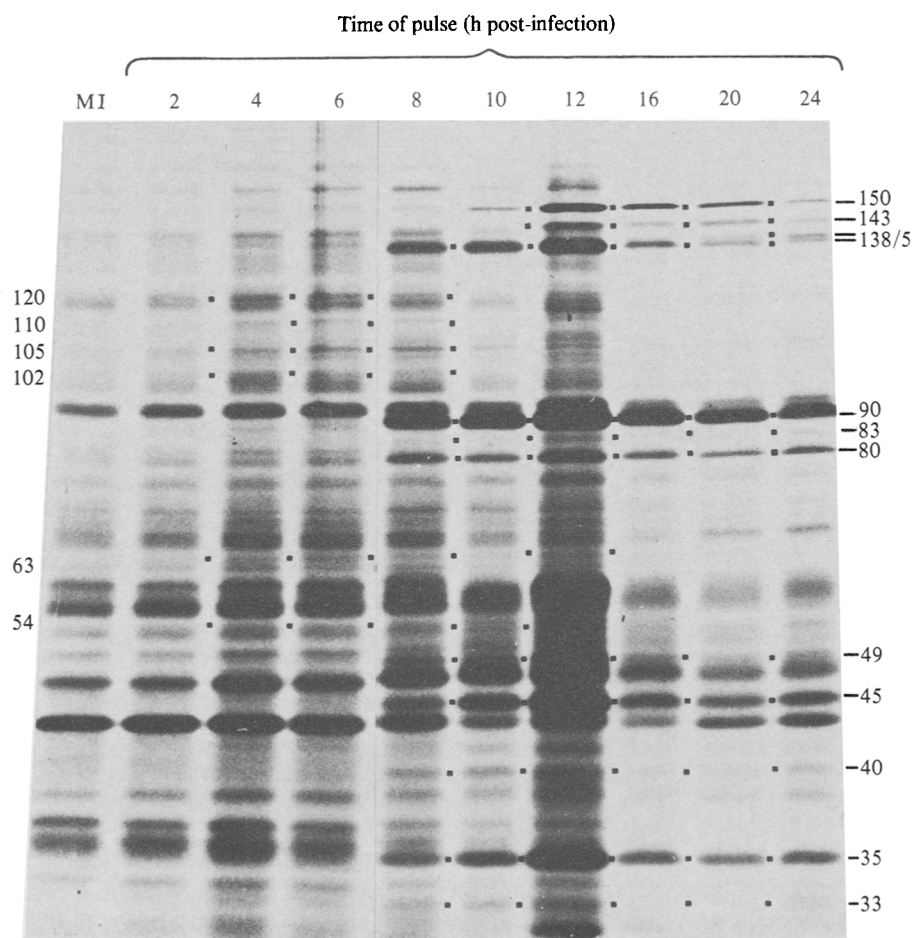


Fig. 1. PAGE analysis of proteins synthesized in superinfected Raji SI cells at different times after infection. The infected cells were labelled at the times indicated above the tracks and prepared for PAGE as described in Methods. A mock-infected sample is labelled MI. Proteins induced by superinfection are indicated by a dot at the left of the track. The numbers referring to the virus-induced proteins are their mol. wt. $\times 10^{-3}$. Approx. $65 \mu\text{g}$ protein were applied to each slot. Due to the decrease in incorporation of [^{35}S]methionine late after infection it was necessary to prepare two autoradiograms from the same gel and cut and reassemble them to allow identification of proteins at late times after infection.

Effect of cycloheximide inhibition on the synthesis of virus-induced protein synthesis

CH was added to Raji SI cells at various times after infection. At 12 h post-infection the drug was washed from the cultures and the cells were pulse-labelled (30 min) in the presence of actinomycin D. From the data presented in Fig. 2 it is apparent that when superinfected cells were treated with CH at 0 or 2 h post-infection that after removal of the drug six virus-induced proteins were observed (proteins 120, 110, 105, 102, 63 and 54). When CH was added at 6 or 8 h post-infection subsequent release of the block led to the synthesis of additional proteins 150, 138, 90, 80, 49, 45, 40, 35 and 33, although in lesser amounts than in untreated cultures and it was also noticeable that the synthesis of proteins 120, 110, 105, 102, 63 and 54 had decreased. When the drug was added later than 10 h post-infection little effect was noticed except that proteins 143 and 83 were made in reduced amounts. These observations suggest that the virus-induced proteins belong to three groups: primary proteins

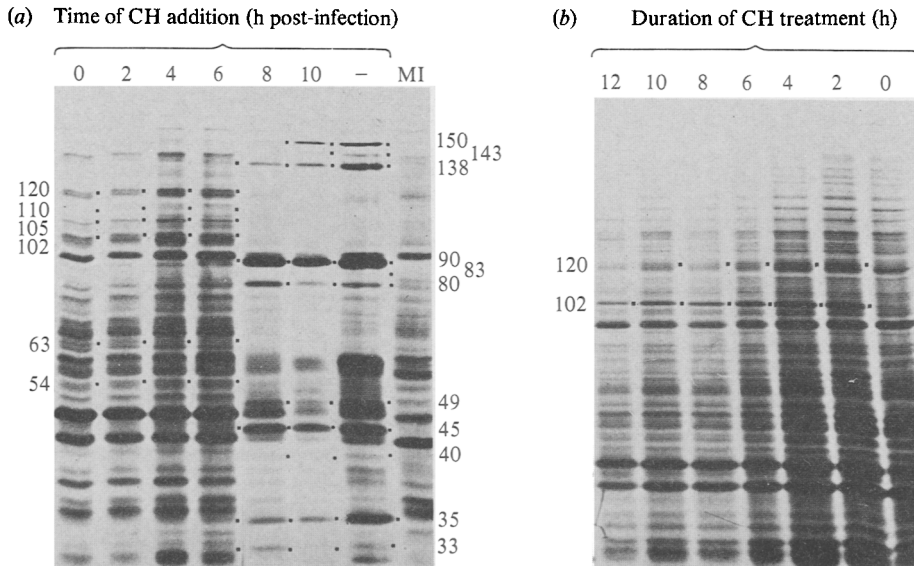


Fig. 2. (a) Effect of cycloheximide (CH) on the synthesis of proteins in Raji SI cells. CH was added to cultures of Raji SI cells at the times indicated and washed from the cultures at 12 h post-infection (in the presence of actinomycin D). The samples were labelled and prepared as described in Fig. 1. (b) Effect of cycloheximide (CH) treatment on the synthesis of proteins in uninfected Raji cells. CH was added to a culture of uninfected Raji cells and samples were removed at various times. The duration of the CH block is given above the tracks in this figure. The cells were pulse-labelled and prepared for PAGE. The two CH-inducible proteins are indicated by dots placed to the left of the tracks. Approx. 65 μ g protein were applied to each slot. Overexposure of the gel did not reveal additional bands.

that can be synthesized in the absence of prior protein synthesis; secondary proteins that require the prior synthesis of proteins but once their synthesis is under way that of proteins begins to decline; tertiary proteins, the synthesis of which is initiated next, but does not appear to preclude further synthesis of secondary proteins.

Treatment of uninfected Raji cells with CH lead to the synthesis of two proteins which are also induced by superinfection (120, 102) (Fig. 2*b*). This observation may indicate that CH partially induced the endogenous EBV genomes.

Analysis of virus-induced proteins by immunoprecipitation

Fig. 3(a) illustrates the proteins precipitated from Raji SI cells by sera containing antibodies against the various EBV antigens. The titres against the various antigen groups are shown in the figure. Cells were labelled from 12 to 16 h post-infection and then prepared for immunoprecipitation as described in Methods. The three profiles presented in Fig. 3(a) were selected from 16 immunoprecipitates prepared with different sera. Serum No. 7070 precipitates all proteins so far found to be accessible for immunoprecipitation in extracts of Raji SI cells. Other sera containing antibodies against VCA and EA (EA⁺VCA⁺ sera) precipitated subsets of this population (data will be presented elsewhere). From this figure it is possible to conclude that part of the 138/135 and part of the 90/88 complexes are specifically precipitated by EA⁺VCA⁺ sera as are proteins 80, 70, 63, 45, 40, 38, 37 and 31. It is not possible to define members of the VCA complex since all the sera used in this study were also positive for early and late membrane antigens (EMA and LMA). The precipitates prepared using a negative control serum (ND) contained so few counts that it was necessary to overexpose the gel by a factor of 5 in order to detect the non-specifically precipitated proteins. In Fig. 3(b) an immunoprecipitation of proteins from Raji SI cells labelled early (2 to 6 h) or

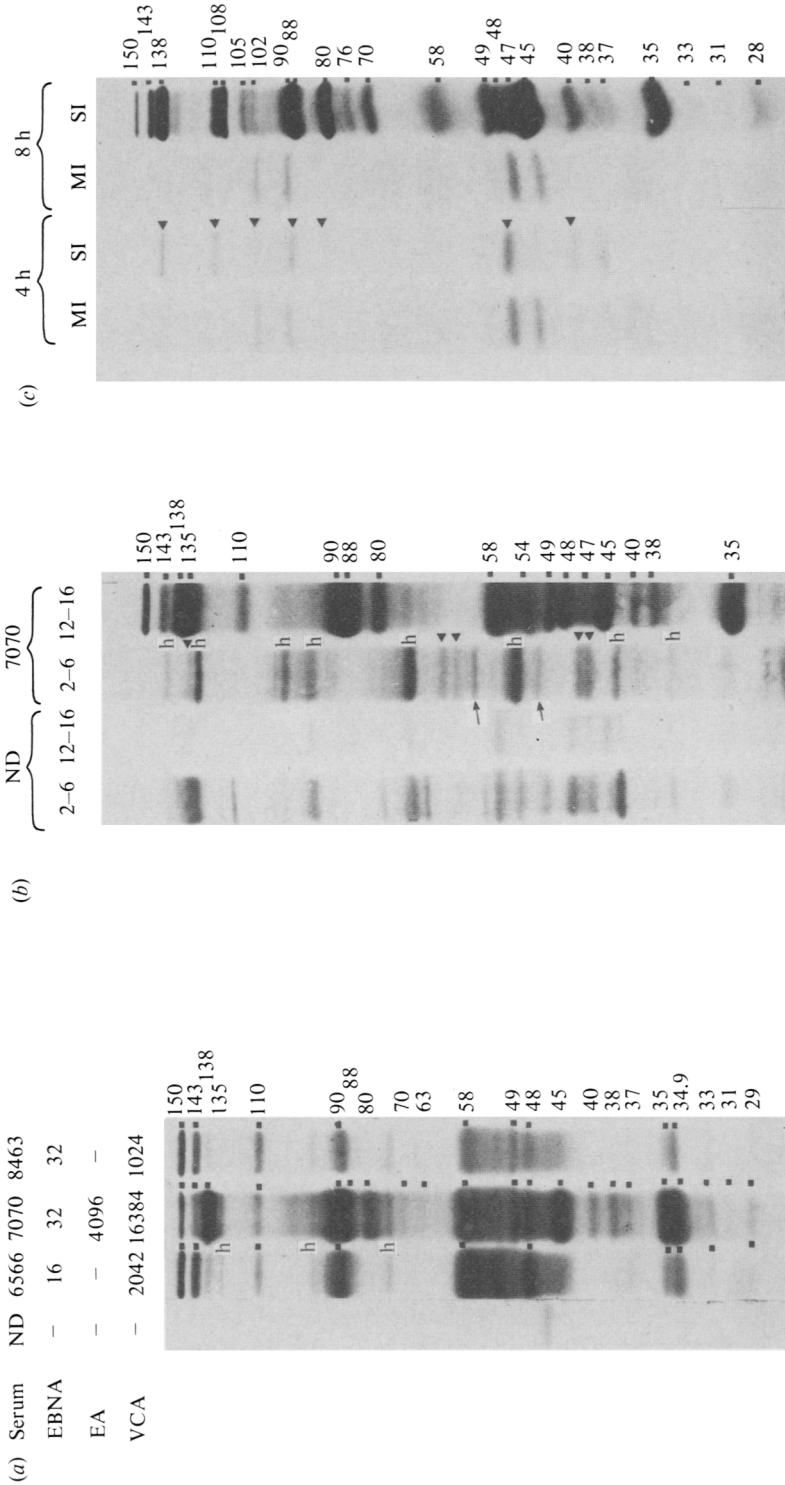


Fig. 3. Immunoprecipitation of EBV-specific polypeptides with human sera containing antibodies against various EBV-determined antigens. (a) Identification of polypeptides specifically precipitated by EA⁺VCA⁺ sera; the titres against the various antigens are shown above the figure. Raji SI cells were labelled from 12 to 16 h post-infection and then processed for immunoprecipitation analysis as described. The negative control sample (SI cells + negative serum ND) contained 400 ct/min in 50 μ l and the three positive immunoprecipitates between 10⁴ and 2 \times 10⁴ ct/min/50 μ l. (b) Identification of proteins synthesized at early (2 to 6 h post-infection) and late (12 to 16 h post-infection) times after infection in immunoprecipitates prepared using serum 7070 or negative serum (ND). Both early labelled samples contained 10⁴ ct/min/50 μ l, ND late 2 \times 10³ ct/min/50 μ l and the 7070 late 3 \times 10⁴ ct/min/50 μ l. (c) Identification of proteins synthesized immediately after release from a cycloheximide block applied at 4 or 8 h post-infection. The precipitates were prepared using a pool of sera having antibodies directed against all EBV-specified antigens (see Methods). Both MI samples and early SI sample contained 10⁴ ct/min/50 μ l, and the late SI sample contained 3 \times 10⁴ ct/min/50 μ l. Specifically precipitated proteins are indicated by a dot, host proteins by h and early synthesized virus-induced proteins by a triangle.

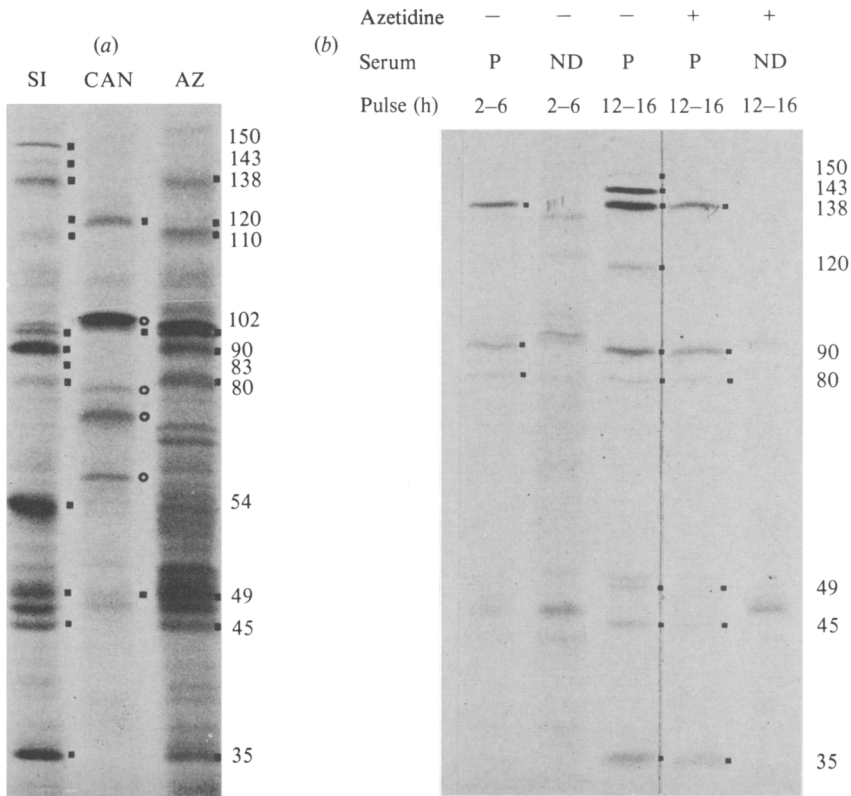


Fig. 4. Effect of amino acid analogues on the synthesis of virus-induced proteins in Raji cells. (a) Canavanine (CAN) or azetidine (AZ) were added to Raji SI cells at 0 h post-infection, the cells were labelled at 12 h post-infection and prepared for PAGE analysis as described. Proteins normally seen in Raji SI cells (track SI) are indicated with a dot and proteins appearing only after treatment with canavanine are indicated by an open circle. Approx. 65 μ g protein were applied to the gel. (b) Immunoprecipitation of proteins from Raji cells treated with azetidine. Raji cells were labelled from 2 to 6 or 12 to 16 h post-infection, azetidine-treated cells from 12 to 16 h post-infection and immunoprecipitates prepared using negative (ND) or positive (P) sera. The samples (in order of appearance in the figure) contained 5×10^2 , 2×10^2 , 10^4 , 10^3 and 10^2 ct/min/50 μ l. Specifically precipitated proteins are indicated by a square to the left of the profiles.

late (12 to 16 h) after infection (using serum 7070) is presented. Immunoprecipitates from the samples labelled at early times after infection contained many more non-specifically precipitated proteins than the samples labelled late in the infectious cycle. This is probably due to the inefficient inhibition of host protein synthesis at early times after infection. Several of the proteins synthesized at early times during the infectious cycle and precipitated by serum 7070 were identified in Fig. 3(a) as being specifically precipitated by EA⁺VCA⁺ sera (138, 70, 63 and 47). Comparison of these results with the results presented in Fig. 2(a) suggests that most of these polypeptides are secondary proteins. We therefore prepared immunoprecipitates from cells labelled immediately after release from a CH block applied at 4 or 8 h post-infection. Fig. 3(c) illustrates the results from such an experiment, in which we used a pool of EA⁺VCA⁺ antisera.

These profiles confirm the supposition that proteins specifically precipitated with anti-EA-positive sera belong to the group of secondary proteins (138, 90, 80, 47, 40) with the

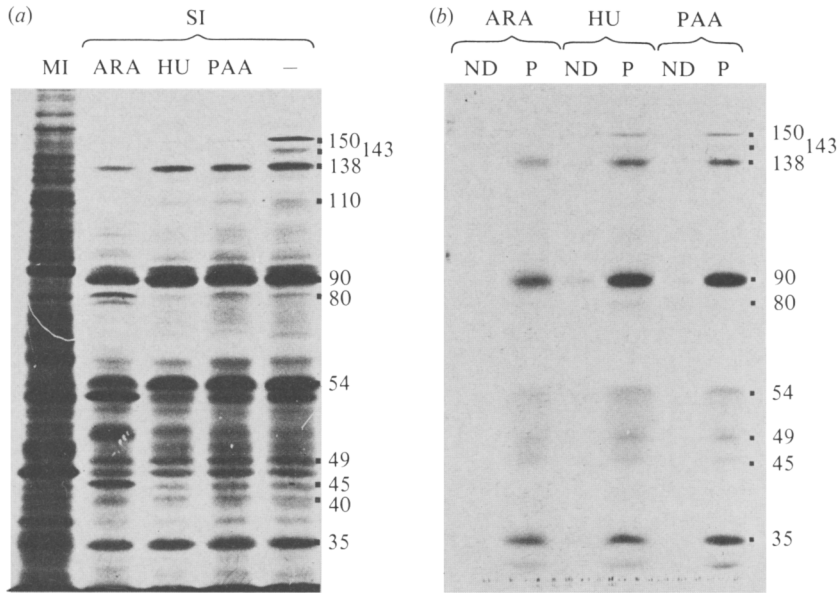


Fig. 5. Effect of inhibitors of DNA synthesis on the synthesis of virus-induced proteins in Raji SI cells. (a) MI, Uninfected Raji cells; SI, superinfected Raji cells, —, no inhibitor; ARA, cytosine arabinoside, HU, hydroxy-urea; PAA, phosphonoacetic acid. The inhibitors were added to the cells at the time of infection and the cells labelled at 12 h post-infection (in the presence of inhibitor) and prepared for PAGE as described. Virus-specified proteins are indicated by a dot to the left of the untreated SI track. (b) Immunoprecipitates prepared from SI cells treated with the inhibitors of DNA synthesis using negative serum (ND) or positive sera (P). The immunoprecipitation contained (in order of appearance in the figure) 1.5×10^2 , 1.5×10^3 , 2×10^2 , 8×10^3 , 8×10^2 , 14×10^3 ct/min/ 50μ l.

exception of protein 63 which is a primary protein. Note also that CH-inducible protein 102 is also precipitated from CH-treated, uninfected Raji cells by this pool of serum.

Effect of amino acid analogues on the synthesis of virus-induced protein synthesis

In the preceding experiments we demonstrated an apparent relationship between three groups of virus-induced proteins with respect to their coordinated synthesis. In order to test the hypothesis that the control elements are in fact virus-induced proteins and not some other factor, it is necessary to observe the effect of synthesis of defective primary proteins on the synthesis of secondary and tertiary proteins. To this end Raji cells were superinfected in the presence of either canavanine or azetidine and incubated with the drug until 12 h post-infection, at which time the cells were labelled in the presence of the analogue. The population of proteins synthesized under these conditions was analysed on SDS-polyacrylamide gels and the result of one such experiment is presented in Fig. 4(a). The presence of canavanine prevented the synthesis of most proteins with the exception of three proteins (120, 102 and 49). In addition to these three proteins a number of previously unidentified proteins were observed (marked with open circles in Fig. 4a). The origin of these remains obscure; they may represent proteins which were not correctly processed (cleavage, glycosylation, phosphorylation or sulphation) due to the presence of the amino acid analogue. Alternatively, they may be primary proteins which were not previously identified due to insufficient accumulation in the absence of canavanine.

Azetidine, although it inhibits the synthesis of many virus proteins does not have the drastic effect of canavanine, since nine proteins are synthesized in its presence (138, 120, 110, 102, 90, 80, 49, 45 and 35). Since treatment of the cells with amino acid analogues reduces

Table 1. Summary of the EBV-induced proteins

PP*	Observed		Specifically precipitated by EA ⁺ sera	Time of maximum synthesis (h post-infection)	Sensitivity to CH block §	Sensitivity to inhibition with				
	DIR †	IP ‡				PAA	HU	AraC	CAN	AZ
150	•	•		12-16	2	±S	±S	S	S	S
143	•	•		12	3	±S	±S	S	S	S
(138) ¶	•	•	•••	8-12	2	NS	NS	NS	S	NS
(135) ¶	•	•								
120	•	•		4-8	1				NS	NS
110	•	•		4-8	1	NS	NS	NS	S	NS
108		•								
105	•	•		4-8	1					
102	•	•		4-8	1				NS	NS
(90) ¶	•	•	•••	8-24	2	NS	NS	NS	S	NS
(88) ¶	•	•		8-24	2					
83	•	•		8-24	3				S	S
80	•	•	•	8-24	2	NS	NS	NS	S	NS
76		•								
70		•	•							
63	•	•	•	4-8	1					
58		•								
54	•	•		4-8	1	NS	NS	NS	S	S
49	•	•		8-24	2	NS	NS	NS	NS	NS
48		•								
47		•								
45	•	•	•	8-24	2	NS	NS	NS	S	NS
40	•	•	•	8-12	2					
38		•	•							
37		•	•							
(35) ¶	•	•	•••	8-24	2	NS	NS	NS	S	NS
(34.5) ¶	•	•			2					
33	•	•		8-12	2					
31		•	•							
29		•								

* Polypeptide; the numbers used to identify the various polypeptides are the apparent mol. wt. $\times 10^{-3}$.

† Observed in total cell extracts analysed directly in SDS polyacrylamide gels.

‡ Only seen after enrichment by immunoprecipitation.

§ 1, Primary protein; 2, secondary protein; 3, tertiary protein.

|| PAA, phosphonoacetic acid; HU, hydroxy-urea; AraC, cytosine arabinoside; CAN, canavanine; AZ, azetidine; S, sensitive to inhibitor; NS, not sensitive; \pm S, synthesis of the polypeptide is considerably reduced in the presence of the inhibitor.

¶ These bands contain more than one polypeptide, 138/135 is a triplet containing two virus-induced proteins and one host protein. The bands 90/88 and 35/34.5 are doublets containing two virus-induced proteins.

** EA specificity resides in one member of the multiple band.

the synthesis of total protein considerably, immunoprecipitates were prepared from treated cultures in order to detect proteins synthesized in small amounts. The results from such an experiment using azetidine-treated cells failed to reveal any additional proteins (Fig. 4*b*). The Raji SI cells treated with canavanine remained negative in the indirect fluorescent antibody staining test (FA test) for EBV, EA or VCA whereas those treated with azetidine were positive for EA.

Dependence of virus-induced protein synthesis on DNA replication

Raji SI cells were treated with either cytosine arabinoside (AraC), hydroxyurea (HU) or phosphonoacetic acid (PAA) as described in Methods from 0 to 12 h post-infection. The cells were then pulse-labelled in the presence of the inhibitors. The results of such an experiment are presented in Fig. 5(*a*). From these profiles it is apparent that inhibition of DNA synthesis with PAA or HU prevents the synthesis of the tertiary proteins and, in addition, reduces the

quantity of protein 150, traces of which can be detected in Fig. 5 (*a*). Analysis of the samples by immunoprecipitation (Fig. 5*b*) revealed that proteins 150 and 143 are synthesized, although in reduced amounts. Inhibition of DNA synthesis with AraC has similar effects; however, its inhibitory activity appears to be more complete since proteins 150 and 143 could not be detected even after immunoprecipitation. All Raji SI cells treated with the inhibitors were positive for EA but negative for VCA in the FA test. Table 1 summarizes all the data presented in these experiments.

DISCUSSION

The sequential synthesis of virus-induced proteins observed in the experiment presented in Fig. 1 suggested to us that the synthesis of proteins induced by EBV within Raji SI cells may be coordinately regulated by mechanisms similar to those described by Honess & Roizman (1974, 1975), Rakusanova *et al* (1971) and Wolf & Roizman (1977) in HSV-infected cells. To test this hypothesis we carried out experiments using cycloheximide, canavanine and azetidine (Fig. 2 and 4) and inhibitors of DNA replication. One major difference between the Raji SI cell and the HSV-infected cell is that all Raji cells already contain EBV genomes which are partially expressed. It is therefore not justified to assume that the EBV and HSV systems are exactly analogous. For this reason we have refrained from using the alpha, beta, gamma nomenclature introduced by Honess & Roizman for HSV and have defined the three groups of proteins as primary, secondary and tertiary groups. The experiments shown in Fig. 2 (*a, b*) indicate that the synthesis of secondary proteins is dependent upon the prior synthesis of primary proteins, and once the synthesis of secondary protein is under way, the synthesis of primary proteins is inhibited. Similarly, the synthesis of tertiary proteins is dependent upon the prior synthesis of secondary proteins. The synthesis of secondary proteins does not seem to be inhibited by tertiary proteins. Whether EBNA is required for the initiation of primary protein synthesis is not clear from these experiments. EBNA may be a primary protein itself or its synthesis may precede that of primary proteins and its presence may be a requirement for the initiation of further protein synthesis. The fact that the superinfection system is more complex than the lytic cycle following infection with HSV is reinforced by the possible activation of the endogenous EBV genomes after CH treatment. Although immunofluorescent staining could not detect EA in the CH-treated, uninfected Raji cells, the two CH-inducible proteins (120 and 102) identified in Fig. 2 (*a, b*) may be the first steps towards the expression of the immunologically defined EA complex without being part of it. Synchronization of the cells before CH treatment allows the synthesis of EA in uninfected Raji cells. These observations are identical to those reported by Hampar *et al.* (1976) who showed that addition of CH to Raji cells at the beginning of the S phase permitted the synthesis of EA 5 h after the removal of the inhibitor. The observation that the primary proteins appear in subgroups leads to the hypothesis that a sequence of events, specified by EBV, occurs immediately after infection and is essential for subsequent synthesis of EA. Further evidence that the primary phase of protein synthesis can be subdivided comes from the experiments using canavanine which, when added at the time of infection, prevented the synthesis of several but not all of the primary proteins (see Fig. 4 and 6). Full expression of the primary and partial expression of the secondary groups of proteins is observed when canavanine is replaced by azetidine (Fig. 4 and 6). This observation may indicate that more than one control mechanism may operate the switch from primary to secondary synthesis, that one controlling element cannot be made in an active form in the presence of canavanine and that this element is required for full expression of primary proteins. This or these primary protein(s) can be synthesized in the presence of azetidine and permit a partial expression of the secondary group. Whether the full expression of the secondary group requires a second primary protein (which cannot be made in active form in the presence of azetidine) or whether transition from secondary a to secondary b group is controlled by a secondary a protein remains obscure. Addition of inhibitors of DNA synthesis before the onset of viral DNA

replication prevented the normal synthesis of tertiary proteins (Fig. 5). The effect of AraC was more drastic than the effect of PAA or HU. With AraC proteins 150 (which according to other criteria is a secondary protein) and 143 were not synthesized; PAA and HU permitted a very reduced synthesis of these two proteins. This difference in effect may be explained by the mode of action of the three inhibitors: PAA inhibits the replication of the infecting viral genomes but not of host or latent EBV genomes, HU on the other hand appears to prevent host and latent EBV DNA replication whilst permitting replication of the EBV genomes during productive infection, and AraC prevents all DNA replication (Bayliss & Nonoyama, 1978; Mele *et al.*, 1974; Yajima *et al.*, 1976). Thus, if the mRNA for proteins 150 and 143 is transcribed from virus DNA with low efficiency, or the mRNA has either a short half-life, inefficient processing potential or low ribosome-binding capacity, then reduction in the amount of viral DNA available for transcription will have a greater effect upon the synthesis of these two proteins when compared to others; this would correspond with observations in the HSV system (Wolf & Roizman, 1977). As the pool of virus DNA increases the amount of mRNA for proteins 150 and 143 available for translation will also increase, and since either HU and PAA have only a partial effect on total viral DNA synthesis within the superinfected Raji cell it might be expected that small amounts of these proteins would be observed in the cells treated with these two inhibitors. The observation that inhibition of DNA synthesis prevents the normal transition from secondary to tertiary protein synthesis may indicate that the protein responsible for the switch acts indirectly by stimulating virus DNA synthesis (or in fact be the virus-coded DNA polymerase) rather than acting directly upon the viral genome as a regulatory protein to initiate transcription of a 'late' region. The results from the CH experiments or experiments done with amino acid analogues cannot differentiate between these two possibilities since addition of CH before the synthesis of a significant amount of viral DNA or synthesis of a defective DNA polymerase due to the presence of amino acid analogues may directly affect DNA synthesis as well.

Immunoprecipitation experiments with Raji SI cells (Fig. 3, 4*b*, 5*b*) indicate that proteins specifically precipitated by EA⁺VCA⁺ sera (138, 90, 80, 63, 45, 40, 38, 37 and 31) are members of the secondary protein group, with one exception, pp63, which is a primary protein. These results differ from those published by Mueller-Lantzsch *et al.* (1979) who have described five polypeptides specifically precipitated by anti-EA sera (having apparent mol. wt. of 120×10^3 , 85×10^3 , 35×10^3 and 16×10^3). These probably correspond to our proteins 138, 90 and 35, since the two proteins with lower mol. wt. would not have been resolved in our gel system. The reason for the additional proteins we have observed may have been the use of sera with differing specificities for the various components of the EA complex. The sera used by Mueller-Lantzsch *et al.* (1979) may have contained antibodies against a subset of the EA complex. This contention is supported by our unpublished observation that not all EA⁺VCA⁺ sera precipitate the full spectrum of EA proteins, and by the observations of Henle *et al.* (1971) who demonstrated that sera derived from patients with different EBV-induced diseases have different specificities against subcomponents of the EA complex in the fluorescent antibody staining test. From the data presented it is apparent that the regulation of EBV-induced protein synthesis is complex and a classification into primary, secondary and tertiary phases by experiments using CH inhibition is certainly not detailed enough. The first two groups can be subdivided on the basis of the results of experiments using amino acid analogues and inhibitors of DNA replication. Fig. 6 gives a summary of the data and shows the regulatory steps so far identified. Consideration of the immunoprecipitation analysis and the reported division of the EA complex into two components by fluorescent antibody staining (Henle *et al.*, 1971) suggests that phase 2 may be further subdivided. Indeed Mueller-Lantzsch *et al.* (1979) have shown that in phorbol ester-treated Raji cells only a partial expression of the EA complex occurs (proteins 85 and 35) whereas cells which spontaneously produce virus (P3HR1 and B95) contained the

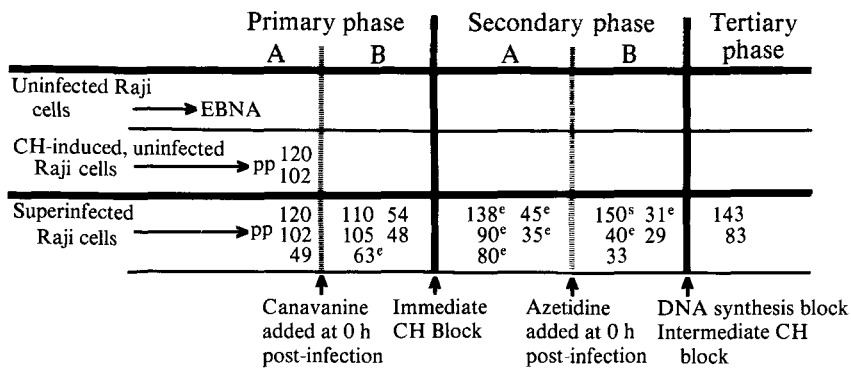


Fig. 6. A model for the sequence of events in Raji SI cells. The three major phases of protein synthesis (primary, secondary and tertiary) are defined by the response of the superinfected cells to cycloheximide blocks applied at different times after infection. The primary and secondary phases are further subdivided according to the effect of amino acid analogues. Proteins marked with a superscript e are specifically precipitated by EA⁺ sera. Protein 150 (s) is partially sensitive to inhibition by inhibitors of DNA synthesis, although it is synthesized immediately after release from an intermediate CH block. The status of EBNA within this scheme remains to be clarified.

remaining components of the EA complex which they were able to identify. Whether this partial expression of EA is due to a defective endogenous genome which does not contain the information for the complete EA complex, or whether those EA proteins synthesized in induced Raji cells are defective and unable to induce the synthesis of the remaining EA proteins is not clear. These possibilities are presently under investigation.

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Note added in proof: during the process of submission of this paper R. J. Feighney, M. P. Farrell and J. S. Pagano published a paper extending our previous finding on kinetics and effects of inhibition of DNA synthesis to 29 proteins. They, in addition, give data on phosphorylation and cellular compartmentalization of the virus-induced cells (*Journal of Virology*, 1981, **37**, 204–207).

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