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Biochemistry

Recovery of *myc*-specific sequences by a partially transformation-defective mutant of avian myelocytomatosis virus, MC29, correlates with the restoration of transforming activity

(viral and cellular oncogenes/hematopoietic cell transformation/recovered virus)

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ABSTRACT **Avian myelocytomatosis virus MC29 transforms fibroblasts and macrophages *in vitro*. Recently we isolated three deletion mutants of MC29 that have a decreased ability to transform macrophages while retaining their capacity to transform fibroblasts. One of these mutants, MC29 *td*10H, on passage through chicken embryo cultures gave rise to a recovered virus MC29 10H B1, which has regained the ability to transform macrophages efficiently. Immunoprecipitation analysis of MC29 10H B1-infected cells revealed a 108,000-dalton *gag–myc* polyprotein as opposed to the 90,000-dalton protein of MC29 *td*10H or the 110,000-dalton polyprotein of *wt*MC29. Tryptic peptide mapping studies demonstrated that the 108,000-dalton protein had acquired v-*myc* peptides that were lost from the *td*10H 90,000-dalton polyprotein and two novel peptides. Restriction enzyme analysis of the MC29 10H B1 proviral DNA also showed that *myc* sequences had been acquired. These results suggest that MC29 *td*10H has recombined with c-*myc* sequences to generate a recovered virus, MC29 10H B1.**

The avian acute leukemia virus MC29 has been shown to induce a variety of tumors in chickens, including renal and hepatic tumors (endotheliomas and carcinomas), sarcomas, and myelocytomatosis (1, 2, 3), whereas *in vitro* MC29 appears to transform both fibroblasts and macrophages. The virus itself was shown to be replication defective, having partial deletions in the *gag* and *env* genes, and was shown to contain specific sequences termed *myc* (4, 5, 6). The only detectable protein product encoded by MC29 is a polyprotein of 110,000 daltons (p110) that contains peptides from the *gag* region and the *myc*-specific sequences (7, 8, 9).

Definition of *myc* as the *onc* gene of MC29 was accomplished recently by using a series of nonconditional partially transformation-defective mutants of MC29 termed *td* 10A, *td* 10C, and *td* 10H. These mutants can transform fibroblasts but have a decreased ability to transform hemopoietic cells *in vitro* and to induce tumors *in vivo* (10, 11). Biochemical analysis showed that they encoded smaller *gag*-related polyproteins, which tryptic peptide analysis revealed had lost *myc*-specific peptides (12). In addition, analysis of the viral RNA by oligonucleotide mapping and proviral DNA by restriction mapping (13, 14) has shown that the mutants have sustained deletions in the *myc* gene, implying that these regions are important in determining the oncogenic properties of the virus *in vitro* and *in vivo*.

It appears likely that the *myc* gene also can exert an oncogenic effect as a cellular gene (c-*myc*; 15, 16) because enhanced expression of c-*myc* mRNA has been found in bursal lymphomas in chickens infected with avian leukosis virus (17, 18).

In this paper we describe the isolation of a virus derived after *in vitro* passage of the MC29 deletion mutant *td*10H in macrophages. This new virus, designated MC29 (in short, HB1), resembles wild-type MC29 in that it can transform macrophages. In addition, biochemical characterization of this new virus revealed that it has regained *myc*-specific information, presumably from the c-*myc* gene.

MATERIALS AND METHODS

Viral Transformation Assays. The Methocel assay and the focus assay in chicken embryo cells (consisting of both fibroblasts and macrophages) has been described earlier (19, 20). Titers are expressed in focus-forming units per ml (FFU/ml).

Clone Purification of HB1 Virus. Nonproducer quail fibroblasts transformed by *td* 10H (10) were superinfected with ring necked pheasant virus (RNPV) and virus was harvested 5 days later. This stock then was used to transform either chicken bone marrow cells or quail fibroblasts at a low multiplicity of infection. Colonies or foci, respectively, were picked with a drawn-out Pasteur pipette and were propagated until about 1×10^6 cells were obtained. In this way, one bone marrow-derived colony (C2) and nine quail embryo fibroblast-derived foci (Q1–Q9) were obtained. These cells then were tested for the presence of helper virus by reverse transcriptase assays. Clones C2 and Q1 and Q9 were found to be nonproducer clones; they then were superinfected with about 10^4 infectious units of either RNPV or the avian leukosis virus, *td*B77, and supernatants were harvested 5–6 days later.

Protein Analysis. Immune precipitation and tryptic peptide mapping were carried out as described (12).

Restriction Enzyme Analysis. High molecular weight DNA was prepared, digested with the appropriate restriction enzyme, and electrophoresed on 0.8% agarose gels as described (14). After electrophoresis the DNA was transferred to nitrocellulose filters (21) that then were hybridized according to published procedures. The probes used, specific for v-*myc*, *gag*, or the total MC29 genomes, were as described (22, 23, 24) and were nick-translated by using the Amersham nick-translation kit. All enzymes were purchased from Boehringer Mannheim.

RESULTS

Isolation of a Derivative of MC29 to *td*10H Capable of Transforming Macrophages *in Vitro*. We have previously described three deletion mutants of MC29 with a drastically decreased macrophage-transforming potential (10, 11, 12). To at-

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Abbreviations: FFU, focus-forming units; RNPV, ring necked pheasant virus; kb, kilobase(s); LTR, long terminal repeat.

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tempt to isolate mutants that have reacquired this property, we assayed RNPV pseudotypes of MC29 *td*10A, *td*10C, and *td*10H for their transforming ability in bone marrow cells by using the Methocel colony assay. In this experiment, no transformed colonies were obtained with MC29 *td*10A-infected cells, 3 small colonies were obtained with MC29 *td*10C-infected cells, and 16 colonies of variable size were obtained with MC29 *td*10H-infected cells. Of the latter, the five largest colonies were isolated and transferred to 35-mm dishes containing quail fibroblasts; only one grew very well (HB1), two grew poorly, and the others did not grow at all (Fig. 1). The cells then were transferred by trypsinization until most fibroblasts had acquired a transformed morphology and the supernatants were tested for their macrophage-transforming ability. Cell-free supernatants of HB1 induced the formation of about 400 colonies per milliliter. These colonies were slightly larger than colonies induced by wild-type MC29. In contrast, the supernatants of the cultures derived from the two other colonies were negative.

The new virus, designated MC29 *td*10H B1 (in short, HB1), was clone-purified in macrophages (clone HB1 C2) as well as in quail embryo cells (clones HB1 Q1 and HB1 Q9). The macrophage- and fibroblast-transforming abilities of these viruses then were determined in comparison with that of the parental Q10H and the grandparental wild-type MC29 virus. As shown in Table 1, HB1 virus has a similar macrophage-to-fibroblast transforming ratio to that of the original wild-type virus, regardless of which helper virus was used to form pseudotypes. This shows that the regained ability to transform macrophages efficiently is a property of HB1 and not of the RNPV helper. However, it is interesting that the foci induced in fibroblasts were smaller and less distinct, particularly when compared to the foci induced by MC29 Q10H virus.

Biochemical Characterization of HB1 Virus. As HB1 behaves biologically *in vitro* like *wt*MC29, in that it transforms fibroblasts and macrophages with equal efficiency, we wanted to analyze the structure of the viral genome and the protein it encodes to determine if a virus had in fact been isolated, which had recovered information necessary for macrophage transformation by passage *in vitro*.

Initially, the *gag–myc* protein product encoded by HB1 was studied. The parental virus *td*10H was shown previously to encode a protein of 90,000 daltons, whereas *wt*MC29 was shown to encode a protein of 110,000 daltons (7, 10). In contrast to both, HB1 was shown to encode a protein of 108,000 daltons. Fig. 2 shows an example of an immunoprecipitation of *wt*MC29 (Q10), *td*10H, and HB1 (RNPV)-infected chicken embryo fibroblast. As can be seen, in HB1-infected cells a *gag–myc* protein of 108,000 daltons was found in addition to proteins encoded by the helper, $Pr76^{gag}$ and $Pr108^{gag-pol}$.

To study the relationship of the 108,000-dalton protein to 110,000-dalton *wt*MC29 and 90,000-dalton *td*10H, two-dimensional tryptic peptide mapping of proteins labeled with [^{14}C]arginine and [^{14}C]lysine was carried out. Fig. 3 shows the [^{14}C]arginine- and [^{14}C]lysine-labeled tryptic peptides of 108,000-dalton HB1, 110,000-dalton *wt*MC29, 90,000-dalton *td*10H, and 108,000-dalton HB1 mixed with 90,000-dalton *td*10H to identify the changes that had occurred between these proteins. A comparison between 108,000-dalton HB1 and 90,000-dalton *td*10H shows that they are very similar. Of the 24 *gag* peptides present in the 90,000-dalton protein, 23 can be detected in the 108,000-dalton protein and 1 peptide, marked with a star, was found to be missing. This suggests that the difference between the 90,000-dalton and 108,000-dalton proteins was not due to the acquisition of additional *gag* gene-encoded amino acid sequences. Previous studies have shown that 90,000-dalton *td*10H has 37 v-*myc*-specific peptides, whereas the 110,000-dalton protein has 42 (12). The v-*myc* peptides lost by the 90,000-dalton protein—namely, 1, 4, 5, 6, and 7—are found in 108,000-dalton HB1. In addition, two v-*myc* peptides (marked a and b) present in the 90,000-dalton protein are missing from the 108,000-dalton protein, and two unique HB1-specific peptides (marked x and y) were detected that did not comigrate with *gag*- or v-*myc*-specific peptides from the 90,000-dalton protein (Fig. 3). Fig. 3 shows these differences pictorally, for the sake of clarity.

Analysis of Proviral DNA. Analysis of the integrated proviruses of *wt*MC29 (Q8), the parental virus *td*10H, and HB1 was carried out by restriction enzyme mapping. The purpose of these studies was two-fold. First, the possibility existed that the cell line *td*10H harbored a silent provirus capable of causing the transformation of macrophages, which, upon selection, was expressed and isolated as HB1. Therefore, we wanted to determine the number of proviruses integrated in the *td*10H cells from which HB1 had been isolated. Second, we wished to compare the structure of the HB1 genome with that of both *wt*MC29 and the parental *td*10H.

Previous analyses of *wt*MC29 and the viral mutants, including *td*10H, had shown that the restriction enzyme *Eco*RI cut the viruses only once (22, 25). High molecular weight DNA from *wt*MC29 and *td*10H was isolated, digested, and analyzed by gel electrophoresis and Southern transfer as described. The probe used in each case was a nick-translated plasmid *pmyc-Pst* I (23)

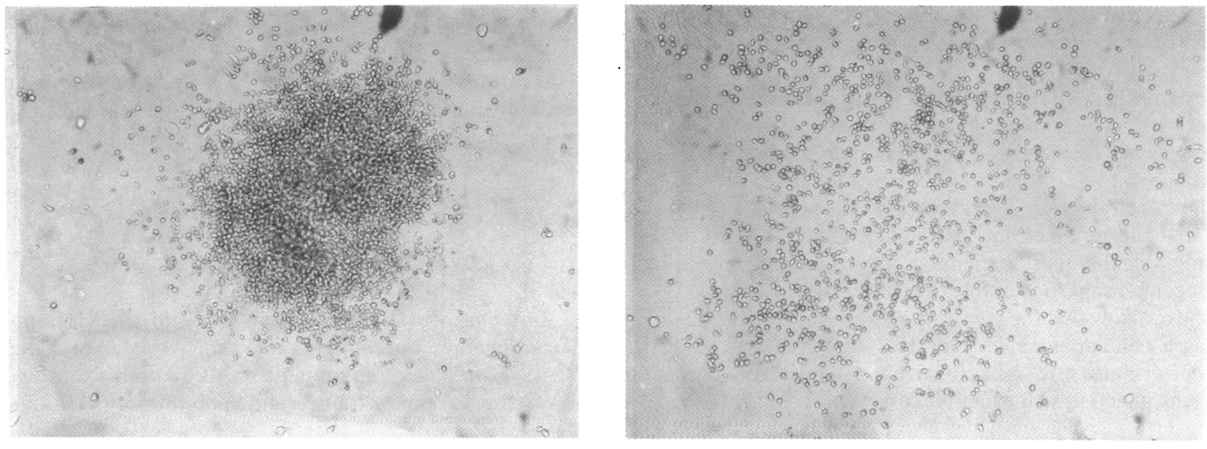

FIG. 1. Macrophage colonies induced by MC29 *td*10H virus. Transformed colonies were isolated and placed in 35-mm dishes containing 1×10^5 quail embryo cells as feeders. Colonies were photographed 7 days after transfer. (*A*) *td*10H clone B1. (×510.) (*B*) *td*10H clone B2. (×510.)

Table 1. Macrophage- and fibroblast-transforming abilities of HB1 virus as assayed on chicken embryo cells

	Macrophage foci, FFU/ml	Fibroblast foci, FFU/ml	Ratio
MC29 HB1 C2 (RNPV)	1.5×10^4	1×10^4	1.5
HB1 Q1 (*td*B77)	1×10^2	5×10^1	2
HB1 Q9 (*td*B77)	2.2×10^2	2.0×10^2	1.1
MC29 *td*10H (RNPV)	6×10^2	7.5×10^5	0.0008
MC29 *td*10H (*td*B77)	$<10^1$	2.5×10^3	0.004
MC29 *wt* (RAV-2)	1.8×10^4	7.5×10^3	2.4

that recognizes most of the *myc* region and a portion of *env*. As can be seen in Fig. 4, only single v-*myc*-specific fragments were found in both cell types; the *Eco*RI fragment of Q8 was 6 kilobases (kb); that of *td*10H was 6.6 kb. An endogenous c-*myc* *Eco*RI fragment of 14 kb was seen in appropriate exposures in each cell line. Because previous analysis of *wt*MC29 and *td*10H by using *Kpn* I, which cuts in the long terminal repeat (LTR), had shown the genome sizes to be 5.5 kb and 4.9 kb (14, 22), respectively, the fragments obtained by *Eco*RI digestion represent junctions with cellular sequences, as predicted for an enzyme which cuts once within the genome. As can be seen, Q8 and *td*10H appear to have only one integrated provirus, eliminating the possibility that the HB1 represents a silent provirus in the *td*10H cell line that is expressed upon selection.

To determine the structure of the HB1 genome, we chose to use a quail cell line transformed by HB1 in the absence of helper virus sequences. Digestion of DNA from these cells with *Eco*RI, probed with the *pmyc-Pst* I plasmid, revealed a 4.2-kb *Eco*RI fragment (as well as an endogenous c-*myc* fragment of 14 kb), which is less than the genome size of *wt* virus or the parental virus *td*10H (Fig. 4). An identically sized *Eco*RI fragment also was found in other HB1-transformed cell lines (data not shown). Taken together, these data implied that *Eco*RI cut more than once within the genome of HB1. If *Eco*RI had cut the genome only once, junction fragments of different sizes would have been expected. Double digestions of HB1 DNA with *Eco*RI and *Pst* I or *Eco*RI and *Bam*HI (data not shown) placed the *Eco*RI sites within the *env* gene and in the viral LTR. To size the HB1 genome, digestions with *Kpn* I were carried out, because *Kpn* I cuts in the LTR of *wt*MC29 and *td*10H (14, 22). However, only fragments >10 kb were observed, suggesting that HB1 had lost the *Kpn* I sites found in the LTR of *wt*MC29 and *td*10H. In contrast, it appears to have gained *Eco*RI sites in the LTR. These data imply that HB1 has a different LTR structure than that which is normally found in MC29 viruses.

Digestion of *wt*MC29 (Q8) with the enzymes *Pst* I and *Bam*HI had shown that the *myc*-specific sequences could be located in a 2.5-kb *Pst* I fragment and an overlapping 3.1-kb *Bam*HI fragment (14). Analysis of *td*10H with these enzymes indicated that it contained a 600-base-pair deletion within each of these fragments, giving rise to a *myc*-specific *Pst* I fragment of 1.9 kb and a *Bam*HI fragment of 2.5 kb. The deletion also appeared to span a *Cla* I site within the *myc* gene of *wt*MC29 (14).

With this information in mind, we wanted to characterize the *myc*-specific sequences of HB1 by using the enzymes *Pst* I, *Bam*HI, and *Cla* I. As can be seen in Fig. 4, when Q8, *td*10H, and HB1 DNAs were digested with *Pst* I, v-*myc*-specific fragments of 2.5 kb, 1.9 kb, and 2.5 kb, respectively, were seen in addition to the c-*myc* fragments of 6.6 kb and 1.1 kb. When digested with *Bam*HI, the v-*myc*-specific fragments detected were 3.1 kb (Q8), 2.5 kb (*td*10H), and 3.1 kb (HB1). Endogenous c-*myc* *Bam*HI fragments were so large that they were excluded

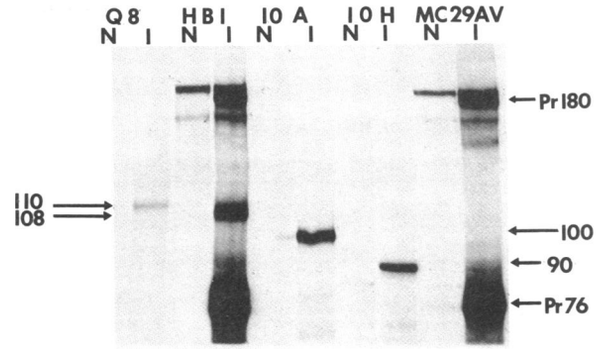

FIG. 2. Identification of the *gag*-related protein of HB1. The *wt*MC29 transformed cell line Q8, HB1 (RNPV)-infected CEF, the nonproducer quail cell lines transformed by *td*10A and *td*10H, and MC29-associated virus (MC29 AV)-infected CEF were labeled for 30 min with 50 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine and the *gag*-related proteins were immunoprecipitated and analyzed by electrophoresis on a 7.5% $NaDodSO_4$/polyacrylamide gel. N, normal rabbit serum; 1, anti-*gag*-serum. Proteins are shown in daltons $\times 10^{-3}$.

from the gel used in this analysis. From these data it appeared that HB1 had regained sequences that gave rise to *Bam*HI and *Pst* I *myc*-specific fragments similar in size to *wt*MC29. Because *Cla* I cuts within the *myc* gene itself, it was of interest to determine if HB1 had regained this *Cla* I site that was lost in *td*10H. Double digests of Q8, *td*10H, and HB1 DNA were carried out with *Bam*HI and *Cla* I. Q8 DNA gave v-*myc*-specific fragments of 1.8 kb and 1.2 kb, indicating that *Cla* I had cut the 3.1-kb *Bam*HI fragment into two fragments; *td*10H DNA gave a 2.5-kb fragment, the size expected if the *Bam*HI fragment was not cut by *Cla* I. However, HB1 resembled Q8, in that two fragments of 1.8 kb and 1.2 kb were observed. This suggests that the *myc*-specific sequences of HB1 resemble the *wt*MC29 *myc*-specific sequences with respect to the enzymes tested, and it implies a reacquisition by *td*10H of *myc* sequences that lead to an increased ability to transform macrophages. The blots shown in Fig. 4 were rehybridized with probes that were representative of the *gag* and the total MC29 genome, and from these data the restriction enzyme map shown in Fig. 4 was derived. For comparative purposes, we also show the restriction enzyme maps of *wt*MC29 and *td*10H determined previously (14). In addition to acquiring *myc*-specific sequences that apparently conserve the characteristic restriction enzyme sites of MC29 v-*myc* gene, the virus appears to have gained *Eco*RI sites in the proviral LTRs as well as new *Pst* I sites in the *gag* gene (Fig. 4).

DISCUSSION

This report describes the isolation and characterization of a virus, HB1, which appears to have been derived from MC29 *td*10H passaged through chicken embryo cells. The virus has regained the ability to transform macrophages, in contrast to *td*10H, and, by the criteria used here (i.e., tryptic peptide mapping and restriction enzyme analysis), has regained *myc*-specific information.

Two mechanisms could be imagined to describe the generation of HB1. The possibility existed that the cell line *td*10H harbored a silent virus that was not expressed until placed under the selective pressure of macrophage transformation. Restriction enzyme analysis seems to rule out this hypothesis because *td*10H contains only one integrated provirus. The unique protein size and restriction enzyme map of HB1 also would rule out the possibility that HB1 represents a *wt*MC29 contaminant of the *td*10H cells at a late date.

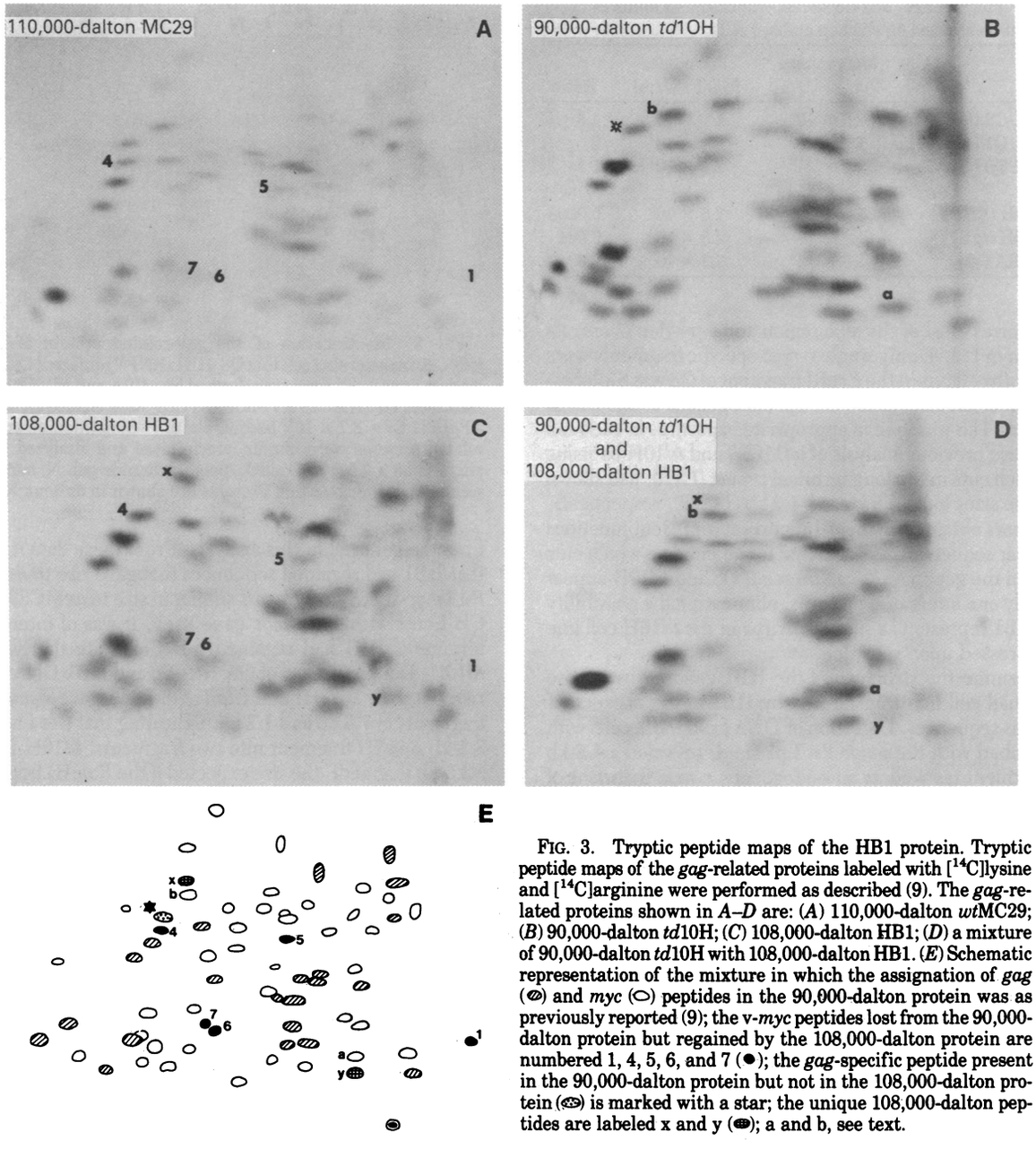

FIG. 3. Tryptic peptide maps of the HB1 protein. Tryptic peptide maps of the *gag*-related proteins labeled with [^{14}C]lysine and [^{14}C]arginine were performed as described (9). The *gag*-related proteins shown in *A–D* are: (*A*) 110,000-dalton *wt*MC29; (*B*) 90,000-dalton *td*10H; (*C*) 108,000-dalton HB1; (*D*) a mixture of 90,000-dalton *td*10H with 108,000-dalton HB1. (*E*) Schematic representation of the mixture in which the assignation of *gag* (⊘) and *myc* (○) peptides in the 90,000-dalton protein was as previously reported (9); the v-*myc* peptides lost from the 90,000-dalton protein but regained by the 108,000-dalton protein are numbered 1, 4, 5, 6, and 7 (●); the *gag*-specific peptide present in the 90,000-dalton protein but not in the 108,000-dalton protein (⊘) is marked with a star; the unique 108,000-dalton peptides are labeled x and y (⊜); a and b, see text.

A more likely postulate is that HB1 represents a recombinant between *td*10H and the endogenous homologue of v-*myc*, c-*myc*. Although this phenomenon has never been observed *in vitro*, others have reported that cellular information (c-*src*) can be recovered *in vivo* by viruses containing deletions in v-*src* (26, 27). In these experiments it was possible to distinguish v-*src* information and c-*src* information in the recovered viruses by tryptic peptide mapping. Analogous studies of HB1 show that the 108,000-dalton *gag–myc* polyprotein contains unique information, in that it has two new tryptic peptides. However, we are unable to relate these unique peptides to c-*myc* because the product of the c-*myc* gene has not been identified to date.

Another way to relate the new HB1 information in HB1 to c-*myc* and to distinguish it from v-*myc* would be to compare the nucleic acid sequence of each. The data available on the structure of the two genes (c-*myc* and v-*myc*) indicate that they are very closely related, sharing *Sac* I, *Sal* I, and *Cla* I sites within analogous regions of the gene (23). Therefore, it is important to note that HB1 has regained a *Cla* I site in a position similar to *wt*MC29. This *Cla* I site is of particular interest because it appears to be at the junction of the exon and intron, which divides the c-*myc* gene into two portions (23). If *td*10H gained c-*myc* information by a recombination event that removed this intron, the *Cla* I site would be left intact as is seen in HB1. A model describing such an event has been proposed by Goldfarb and Weinberg (28). In this regard, it also has been proposed that introns separate functional domains of the protein encoded by the associated exons. One can imagine the *gag–myc* polyprotein of MC29 containing two functional domains, one responsible for fibroblast transformation and one for macrophage transformation, which *td*10H has lost through deletion of a region within the *myc* gene spanning the *Cla* I site. In HB1, it is possible that the virus has acquired from c-*myc* the functional domain (as marked by the *Cla* I site) required for macrophage transformation.

Perhaps the strongest evidence in support of the hypothesis that HB1 is a c-*myc* recombinant comes from a recent preliminary examination of its oncogenic potential. Previous analysis showed that when injected into 1-day-old Brown Leghorn

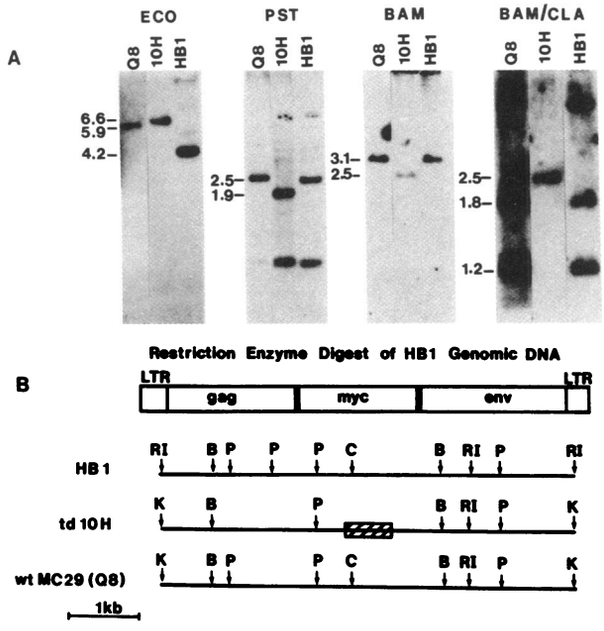

FIG. 4. Restriction enzyme analysis of HB1 proviral DNA. (*A*) High molecular weight DNA that was isolated as described was cleaved with the indicated enzymes; fragments were separated on 0.8% agarose gels by electrophoresis, transferred to nitrocellulose, and probed with nick-translated *pmyc-Pst* I as described (14). The numbers indicate the sizes of the resulting fragments in kb. The DNA was isolated from nonproducer quail embryo fibroblasts transformed by *wt*MC29 (Q8), MC29 *td*10H (10H), or MC29 HB1 (HB1). ECO, *Eco*RI; PST, *Pst* I; BAM, *Bam*HI; CLA, *Cla* I. (*B*) Schematic representation of the restriction enzyme maps of the proviral DNA shown in *A*. The restriction enzyme sites along the proviral genomes are indicated by arrows. The enzymes used were *Eco*RI (RI), *Kpn* I (K), *Bam*HI (B), *Pst* I (P), and *Cla* I (C). The deletion of 0.6 kb in the *td*10H genomes is indicated by the hatched box.

chickens, *wt*MC29 caused predominantly endotheliomas, whereas *td*10H induced no endotheliomas but did cause osteopetrosis and two lymphoblastic lymphomas (11). However, unlike *wt*MC29, HB1 induced lymphomas in most of the birds tested and induced myelocytomatosis in one bird. This disease pattern resembles that induced by avian leukosis virus as a result of proviral insertion in the vicinity of c-*myc*. However, although avian leukemia virus (ALV)-induced lymphomas arise after long latency in the bursa of Fabricius, in the main, lymphomas induced by HB1 arose relatively rapidly without bursal involvement, implying the action of a viral oncogene.

This altered pathology must be characterized more completely. While regaining *myc*-specific sequences, HB1 also has changed in other regards, having gained *Eco*RI sites in the LTR and an additional *Pst* I site in *gag*, perhaps as the result of recombination with helper virus (29). It is important to determine if these changes influence the oncogenic potential of the virus. Molecular cloning of the virus and altering various regions of the genome should establish more precisely the regions required for induction of the diseases observed.

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