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Human cytomegalovirus (strain AD169) contains sequences related to the avian retrovirus oncogene v-*myc*

[herpesvirus/DNA/avian myelocytomatosis virus 29 (MC-29)]

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ABSTRACT **We have detected nucleotide sequences related to the transforming gene (v-*myc*) of avian myelocytomatosis virus MC-29 in the DNA genome of human cytomegalovirus (HCMV) strain AD169. Cloned DNA representing the entire 1.5-kilobase-pair oncogene v-*myc* and subfragments of this gene were hybridized to *Eco*RI-cleaved HCMV virion DNA and cloned subgenomic HCMV DNA fragments. Only a 0.5-kilobase-pair *Pst* I–*Sal* I subfragment representing the 5′ end of the coding sequence of the v-*myc* oncogene hybridized to the HCMV DNA. We have localized these v-*myc*-related sequences to five regions in the long unique segment of the HCMV genome corresponding to *Eco*RI fragments C, I, P, R, and b and to regions within the *Eco*RI junction fragments F and H at or near the repeats bounding the short unique segment. There was no hybridization of these HCMV sequences to other retroviral oncogenes tested including v-*src*, v-*myb*, v-*erb*, v-ST-*fes*, v-*fos*, v-*ras* (Harvey), v-*mos*, and v-*abl*.**

Human cytomegalovirus (HCMV) is a human herpesvirus associated with a wide spectrum of diseases including cytomegalic inclusion disease, mononucleosis, and interstitial pneumonia and disseminated infections in immunosuppressed patients (1). In addition, there is evidence that HCMV has oncogenic properties (2–8) and may play a role in certain types of malignancies (9–14), one of which is Kaposi sarcoma (9, 10). The recent increase in the incidence of acquired immunodeficiency syndrome and Kaposi sarcoma in the United States has focused renewed attention on the oncogenic potential of HCMV.

HCMV strain AD169 has a large linear DNA genome of approximately 240 kilobase pairs (kbp) (15–17). The DNA may be divided into two components designated L (long) and S (short), and each is flanked by inverted repeat sequences (18). The point at which the repeats flanking the L and S segments join is called the L–S junction. We have constructed a cloned library of the *Eco*RI fragments (17) and have mapped these fragments on the genome (18).

In the course of characterizing the HCMV DNA clones we found that several of the viral fragments hybridized to normal human and other eukaryotic DNAs (unpublished data). Retroviruses also contain cell-related DNA sequences which form the oncogenes of the viruses and are referred to as v-*onc*. The cellular progenitors are termed c-*onc* and presumably serve an important functional role in both normal cells and some types of neoplasm (19). The presence of conserved cellular sequences in HCMV, a virus with suspected oncogenic potential, prompted us to determine whether HCMV DNA exhibited homology with any known retroviral oncogene sequences.

In this paper, we report that seven of the HCMV *Eco*RI fragments including C, F, H, I, P, R, and b have homology with v-*myc*, the oncogene from the avian myelocytomatosis retrovirus MC-29.

MATERIALS AND METHODS

Cells, Viruses, Purification of HCMV DNA, and Preparation of Cloned HCMV DNA Fragments. The procedures for growing human embryonic lung cells, infecting the cells with HCMV strain AD169, purifying the viral DNA, and preparing cloned *Eco*RI fragments of the viral DNA have been described (17, 18).

Source of Cloned Retrovirus Oncogenes. The origins of the retrovirus oncogenes subcloned in pBR322 and used in this study are as follows: v-*fos*, 5.8-kbp *Hin*dIII fragment of FBJ murine osteosarcoma virus (20); v-*src*, 0.8-kbp *Pvu* II fragment E of Schmidt–Ruppin A avian sarcoma virus (21); v-*erb*, 0.5-kbp *Pst* I fragment of avian erythroblastosis virus (22); v-*myc*, 1.5-kbp *Pst* I fragment of MC-29 (23); v-ST-*fes*, 0.48-kbp *Pst* I fragment of Snyder–Theilen feline sarcoma virus (24); v-*myb*, 1-kbp *Xba* I–Hae II fragment of avian myeloblastosis virus ligated to *Bam*HI linkers (25); v-*ras*, 0.46-kbp *Sal* I–*Bgl* I fragment of Harvey murine sarcoma virus ligated to *Eco*RI linkers [clone BS-9 (26)]; v-*abl*, 2.3-kbp *Hin*dIII–*Bgl* II fragment of Abelson murine leukemia virus inserted into the *Bam*HI–*Hin*dIII site of pBR322 [clone pAB3 sub 3 (27)]; v-*mos*, 5.9-kbp *Hin*dIII fragment of Moloney murine sarcoma virus [clone pMSV-1 (28)]. DNA samples were generously provided by Rolf Müeller and Inder Verma. The recombinant clone containing the 1.5-kbp *Pst* I fragment from the proviral DNA of MC-29 was supplied by J. Michael Bishop.

Gel Electrophoresis and Hybridization Procedures. DNA fragments cleaved with restriction endonucleases were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (29) or to activated diazobenzyloxymethyl-paper (DBM-paper) according to the supplier's directions (Schleicher & Schuell). Both the nitrocellulose and DBM filters were hybridized as described (18) for 3 days at 37°C to individual DNA fragments labeled with [α-^{32}P]dCTP by nick-translation (30) to 5–50 × 10^7 cpm/μg; 5 × 10^5 cpm of labeled DNA was used per ml of buffer. After hybridization, the filters were washed with 2× standard saline citrate (NaCl/Cit; 1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) at room temperature for 1 hr, with 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$ for 1 hr at 50°C, and with 0.1× NaCl/Cit at room temperature; these are referred to as high-stringency conditions.

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Abbreviations: kbp, kilobase pair(s); HCMV, human cytomegalovirus; MC-29, avian myelocytomatosis virus 29; DBM-paper, diazobenzyloxymethyl-paper; NaCl/Cit, standard saline citrate.

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RESULTS

Hybridization of HCMV *Eco*RI Fragment R to Cloned Retroviral Oncogenes. Several of our cloned HCMV *Eco*RI fragments (17) hybridized to normal cellular DNA sequences that were present in only a few copies per cell (unpublished data). One of these fragments, *Eco*RI fragment R, hybridized strongly not only to human DNA but also to other eukaryotic DNA genomes tested. The transforming genes of retroviruses are also derived from conserved cellular genes. To determine whether HCMV DNA had homology with any known retroviral oncogene sequences, we initially hybridized ^{32}P-labeled HCMV fragment R to a Southern blot of cloned retroviral oncogene fragments including v-*fos*, v-*src*, v-*erb*, v-*myc*, v-*myb*, v-*fes*, v-*ras* (Harvey), v-*abl*, and v-*mos*. When the filters were washed at low stringency (wash with 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$ at 37°C), HCMV fragment R hybridized only to the oncogene v-*myc* (Fig. 1). Washing the filters under high stringency did not reduce the amount of hybridization (data not shown).

The v-*myc* oncogene used in these experiments is contained within a 1.5-kbp *Pst* I restriction endonuclease fragment subcloned from the proviral DNA of the avian acute leukemia virus MC-29. The *Pst* I site, which marks the 5′ end of the subclone, cleaves within the v-*myc* sequence. Thus, this subclone is lacking approximately 160 nucleotides from the v-*myc* sequence (31). *Sal* I cleaves the 1.5-kbp v-*myc* oncogene into two fragments of approximately 0.5 and 1 kbp, corresponding to the 5′ and 3′ ends, respectively, of the coding region. In some experiments the *Sal* I subfragments were used to probe for sequences related to the 5′ and 3′ portions of v-*myc*.

Hybridization of the v-*myc* Oncogene to Cloned *Eco*RI Fragments Representative of the Entire Genome of HCMV. To determine whether sequences related to the v-*myc* oncogene were present in other regions of the HCMV genome, we cleaved each of the 32 recombinant plasmids containing different HCMV subfragments with *Eco*RI. Southern blots of the HCMV DNA fragments were hybridized under stringent conditions to ^{32}P-labeled v-*myc* DNA (Fig. 2). The v-*myc* DNA hybridized to HCMV *Eco*RI fragments C, F, H, P, R, and b. A low level of hybridization to *Eco*RI fragment I was also noted when the autoradiogram was exposed for a longer time. Fragments C, P, R, b, and I are located in the long unique region of the genome. Fragments F and H span the L–S junction of the genome and include sequences from the long repeat, short repeat, and part of the short unique region. These two junction fragments represent the termini of the genome in the inverted orientation such that fragment F equals W + L, and H equals W + N (18). To verify that the presence of v-*myc*-related sequences in the cloned HCMV *Eco*RI fragments was not due to a cloning artifact, we hybridized ^{32}P-labeled v-*myc* DNA to *Eco*RI-cleaved HCMV virion DNA. The hybridization pattern of v-*myc* with virion DNA was consistent with that seen with the cloned HCMV *Eco*RI fragments (data not shown).

Homology Between HCMV and v-*myc* Oncogene Is Localized to the 5′ Coding Sequences of the v-*myc* DNA. To determine the extent of homology between the HCMV genome and v-*myc* oncogene, the 0.5-kbp and 1-kbp *Sal* I DNA subfragments of the v-*myc* oncogene were hybridized individually under stringent conditions to Southern blots of the HCMV *Eco*RI fragments which contained the v-*myc*-related sequences and several negative HCMV fragments. The ^{32}P-labeled 0.5-kbp v-*myc* subfragment hybridized to a Southern blot of each of the positive HCMV *Eco*RI fragments (Fig. 3). However, even with less-stringent conditions, there was no hybridization of the 1-kbp subfragment of v-*myc* to any HCMV fragments (data not shown). These results indicate that the homology between

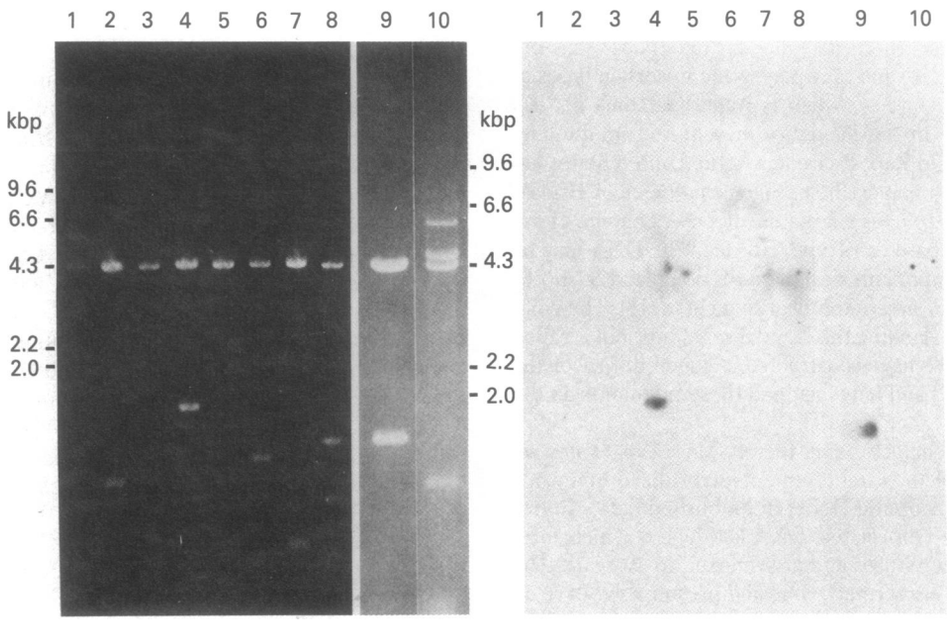

FIG. 1. Hybridization of HCMV *Eco*RI fragment R to v-*myc*. Recombinant DNA clones containing retroviral oncogenes were cleaved with appropriate enzymes to separate the DNA inserts from the plasmids, subjected to electrophoresis on 0.8% agarose gels, and visualized with an ethidium bromide stain (*Left*). *Hin*dIII-cleaved λ DNA provided size markers. The DNA fragments were transferred to nitrocellulose by Southern blotting and hybridized to ^{32}P-labeled purified HCMV *Eco*RI fragment R (*Right*). The filters were washed sequentially as follows: 2× NaCl/Cit, 1 hr at room temperature; 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$, 1 hr at 37°C; and 0.1× NaCl/Cit at room temperature. After washing, the filters were subjected to autoradiography. Lanes: 1, v-*fos* (*Hin*dIII cleavage); 2, v-*src* (*Pvu* II cleavage); 3, v-*erb* (*Pst* I cleavage); 4 and 9, v-*myc* (*Pst* I cleavage); 5, v-ST-*fes* (*Pst* I cleavage); 6, v-*myb* (*Bam*HI cleavage); 7, v-*ras* (Harvey) (*Eco*RI cleavage); 8, v-*abl* (*Sma* I and *Bgl* II cleavage yields two major v-*abl*-containing fragments, 1 and 0.67 kbp); 10, v-*mos* (*Xba* I and *Hin*dIII cleavage yields v-*mos*-containing fragment, 1 kbp; the fragment at 5.9 kbp is an *Xba* I partial digestion product). Lanes 9 and 10 are from a separate gel. The small amount of hybridization to the plasmid band at 4.3 kbp is due to plasmid contamination of ^{32}P-labeled HCMV fragment R.

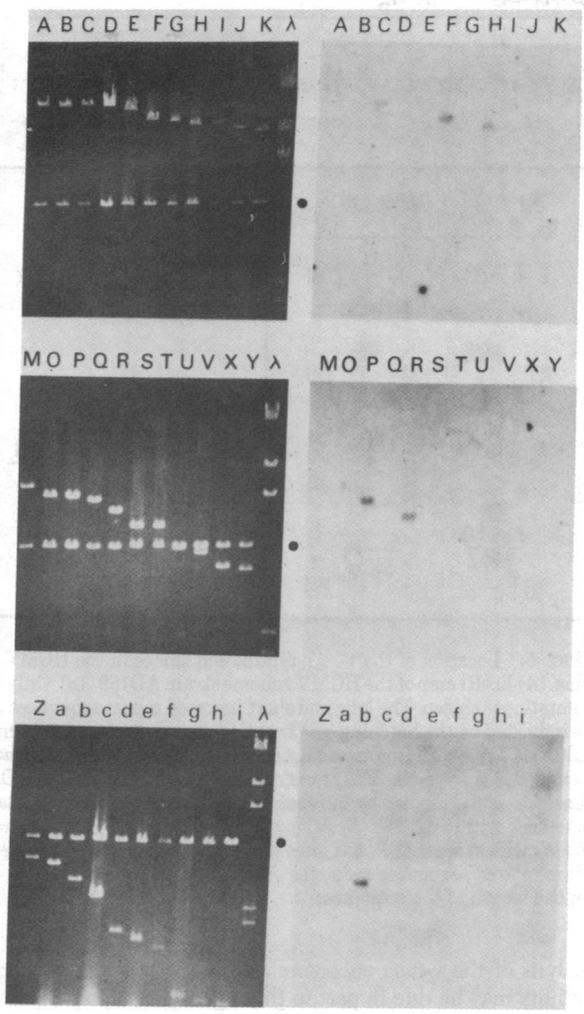

FIG. 2. Hybridization of the ^{32}P-labeled 1.5-kbp *Pst* I fragment of v-*myc* to a Southern blot of HCMV DNA fragments cloned in pACYC 184. Recombinant plasmids were cleaved with *Eco*RI. The resulting fragments were subjected to electrophoresis on 0.8% agarose gels and were visualized with an ethidium bromide stain (*Left*). *Hin*dIII-cleaved λ DNA provided size markers. The DNA fragments were transferred to nitrocellulose by Southern blotting and hybridized to ^{32}P-labeled 1.5-kbp *Pst* I fragment of v-*myc* (*Right*). The filters were washed under high-stringency conditions and subjected to autoradiography. The letters above the lanes correspond to the cloned HCMV *Eco*RI fragments. The position of pACYC 184 is indicated (●).

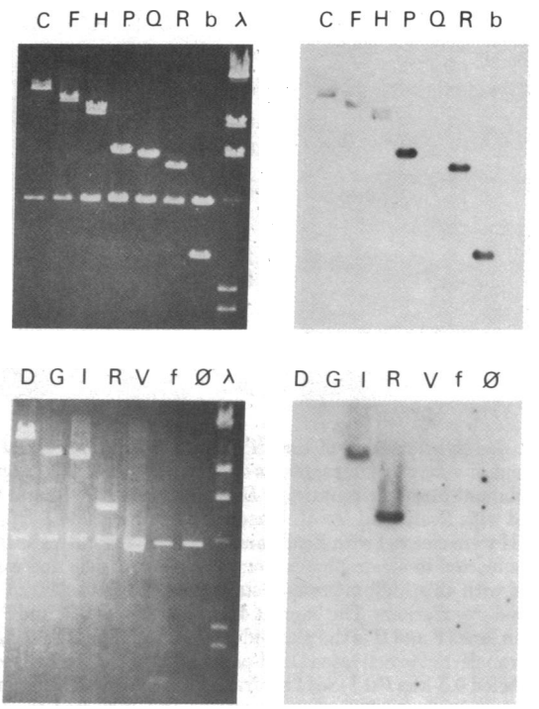

FIG. 3. Hybridization of the ^{32}P-labeled 0.5-kbp *Pst* I–*Sal* I subfragment of v-*myc* to a Southern blot of cloned HCMV DNA fragments. Recombinant plasmids were cleaved with *Eco*RI. The resulting fragments were subjected to electrophoresis on 0.8% agarose gels and were visualized with an ethidium bromide stain (*Left*). *Hin*dIII-cleaved λ DNA provided size markers. The plasmid pACYC 184 (ϕ) is 4.3 kbp. The DNA fragments were transferred to nitrocellulose by Southern blotting (*Right*) and hybridized to the ^{32}P-labeled 0.5-kbp *Pst* I–*Sal* I subfragment of v-*myc*. The filters were washed under high-stringency conditions and subjected to autoradiography. The letters above the lanes correspond to the cloned HCMV *Eco*RI fragments.

HCMV and the v-*myc* oncogene is localized to the 5′ coding sequences of the v-*myc* oncogene.

Localization of v-*myc*-Related Sequences Within the HCMV *Eco*RI Fragments. To localize the v-*myc*-related sequences more precisely within the HCMV genome, we subdivided the positive HCMV *Eco*RI fragments with additional restriction endonucleases of known map position (18) and separated the fragments by agarose gel electrophoresis. The fragments were transferred to activated DBM-paper by blotting and hybridized with the 0.5-kbp 5′ coding subfragment of v-*myc*. A representative blot is shown in Fig. 4 and the data are summarized in Fig. 5.

*Eco*RI fragments R and C both contain v-*myc*-related sequences and are located adjacent to one another on the map. Recently, it has been found that a 2.9-kbp *Xba* I–*Hin*dIII fragment, which maps primarily in the right-hand portion of *Eco*RI fragment R and includes the junction of *Eco*RI fragments R and C, can transform NIH 3T3 cells (6). To determine the location of the v-*myc*-related sequences relative to the transforming region, we subdivided fragments R and C with *Bgl* II (Fig. 4). The *Hin*dIII site marking the right boundary of the transforming region maps within the 5.1-kbp *Bgl* II subfragment of C (part of *Bgl* II fragment Q) and the *Xba* I site marking the left boundary maps within the 3.7-kbp *Bgl* II subfragment of R (also part of *Bgl* II fragment Q). The v-*myc* probe hybridized to the larger 11.3-kbp *Bgl* II subfragment (*Bgl* II fragment M) located in the middle of *Eco*RI fragment C and to the 2.2-kbp *Bgl* II subfragment (part of *Bgl* II fragment L) located in the left-hand portion of fragment R. From these data we concluded that the v-*myc*-related sequences in fragments R and C were not adjoining and did not map within the transforming region defined by DNA transfection.

Both the termini and L–S junction fragments show marked size heterogeneity in the virion (17). The heterogeneity within L–S junction fragments F and H has been mapped to a *Pvu* II fragment (P1 and P11) located at the junction of the long and short repeats (17, 18). The *Pvu* II fragment containing the region of heterogeneity is G+C-rich and contains one stretch of 400 nucleotides which is 74% G+C (D. Filpula, personal communication). To determine the location of the v-*myc*-related sequences with respect to the region of heterogeneity, we hybridized ^{32}P-labeled v-*myc* probe to *Pvu* II fragments from clones representing *Eco*RI fragments F and H (Fig. 4). The v-*myc* probe hybridized to the 4.7-kbp *Pvu* II subfragment (P12) from *Eco*RI fragment F and to the 2.5-kbp *Pvu* II subfragment (P2) from *Eco*RI fragment H. These two subfragments cross-hybridize and map at the junction of the short repeats and short unique

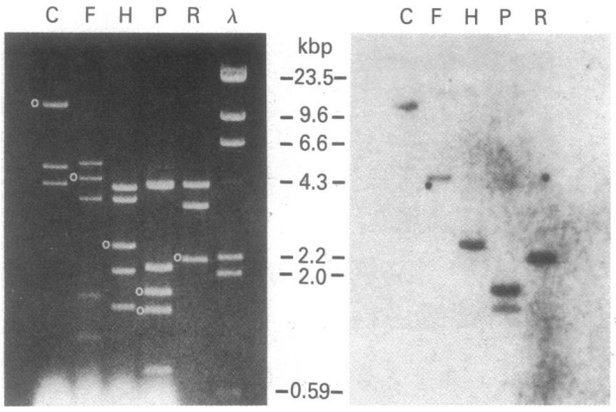

FIG. 4. Hybridization of the ^{32}P-labeled 0.5-kbp *Pst* I–*Sal* I subfragment of v-*myc* to subfragments of cloned HCMV DNA fragments. Recombinant plasmids containing *Eco*RI fragments C, R, and P were cleaved with *Eco*RI and *Bgl* II. Plasmids containing *Eco*RI fragments F and H were cleaved with *Eco*RI and *Pvu* II. The resulting fragments were subjected to electrophoresis on 0.8% agarose gels and were visualized with an ethidium bromide stain (*Left*). *Hin*dIII-cleaved λ DNA provided size markers. The band at 4.3 kbp in lanes C, R, and P and at 4 kbp in lanes F and H is the plasmid pACYC 184. The DNA fragments were transferred to activated DBM-paper (*Right*) and hybridized to the ^{32}P-labeled 0.5-kbp *Pst* I–*Sal* I subfragment of v-*myc*. The filters were washed under high-stringency conditions and subjected to autoradiography. The letters above the lanes correspond to the cloned HCMV *Eco*RI fragments. V-*myc*-containing fragments are shown by ○.

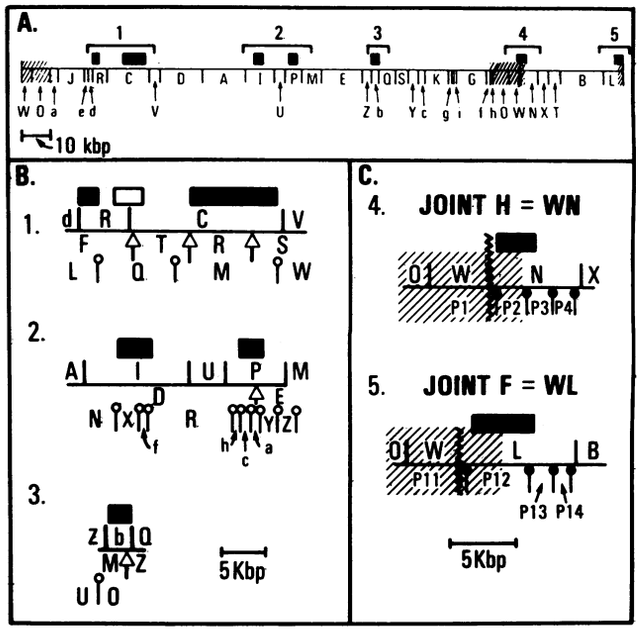

FIG. 5. Location of the v-*myc*-related sequences in the HCMV genome. (*A*) *Eco*RI map of the HCMV genome strain AD169 (18). Only one orientation is shown. The long and short inverted repeat sequences (18) are indicated by the hatching. (*B*) Location of v-*myc*-related sequences within HCMV *Eco*RI fragments R, C, I, P, and b with respect to *Hin*dIII (△) and *Bgl* II (♀) sites. The transforming region as defined by DNA transfection (6) is shown by the open bar. (*C*) Location of v-*myc*-related sequences within HCMV *Eco*RI junction fragment F (L+W) and H (N+W) with respect to *Pvu* II sites (●). The solid bar representing the v-*myc*-related sequences indicates only the approximate location and not the length of the sequences.

region of the genome (18). From these data, we concluded that the v-*myc*-related sequences were contained within the two terminal *Eco*RI fragments in the short region of the genome (L and N in the two orientations) outside the region of heterogeneity.

We have also mapped the v-*myc*-related sequences to the following regions: two adjacent *Bgl* II subfragments a and c (1.6 and 1.2 kbp, respectively) located in the middle of *Eco*RI fragment P (Fig. 4) and to the left-hand region of *Eco*RI fragment b (data not shown for fragment b).

DISCUSSION

In this paper we have shown that several regions of the strain AD169 HCMV DNA genome contain sequences that exhibit homology with the v-*myc* oncogene. We have localized these v-*myc*-related sequences to five regions in the long unique segment of the genome corresponding to *Eco*RI fragments C, I, P, R, and b and to regions within the *Eco*RI junction fragments F and H at or near the repeats bounding the short unique segment (Fig. 5).

Although in the avian retrovirus the *myc* domain is continuous, in both human and chicken cells the endogenous c-*myc* locus consists of two exons separated by a 1-kbp intron. The location of the *Sal* I site in the v-*myc* sequence corresponds to the approximate position of the exon/exon junction in the c-*myc* RNA (32–34). In our hybridization experiments we could detect related sequences in the HCMV genome only with the 0.5-kbp *Pst* I–*Sal* I subfragment of the v-*myc* oncogene. These results indicate that either the HCMV genome contains sequences related only to the 5′ domain of the *myc* gene or that there has been a different degree of evolutionary divergence between the two domains such that the 3′ domain of the HCMV *myc*-related sequences cannot be detected with the avian retrovirus v-*myc* probe. This latter possibility is supported by the observation that heteroduplexes formed between the human c-*myc* gene and avian retrovirus v-*myc* gene show greater stability in the region corresponding to the 5′ exon (32). Sequence analysis of the v-*myc* oncogene also suggests that this greater stability may be due in part to the higher G+C content of the 5′ region (31). Although it is possible that some of the observed homology between v-*myc* and HCMV is a fortuitous consequence of the high G+C contents of both DNAs, it is noteworthy that there is no hybridization of v-*myc* to the G+C-rich *Pvu* II fragment located at the junction of the HCMV long and short repeats.

One question raised by this study concerns the origin of these *myc*-related sequences in the HCMV genome. Has HCMV acquired these sequences from the host cell, and, if so, by what mechanism? Recently Dalla Favera *et al.* (32) reported the isolation of three different recombinant clones containing human c-*myc* sequences that exhibit homology only with the 0.5-kbp *Pst* I–*Sal* I subfragment of v-*myc*. Although these sequences may be part of different genes that are related to only a portion of the c-*myc* locus, it also is possible that these sequences represent pseudogenes analogous to those reported in various eukaryotic species (for review, see refs. 35 and 36). The mechanisms responsible for the generation of pseudogenes remain an enigma. However, if such events can take place with a finite probability, then cytomegaloviruses, which are characterized by persistent and latent infections, may have acquired these sequences during their long interaction with the human host. Comparison of the structure of the *myc*-related sequences and adjacent regions in the cytomegalovirus genome with the endogenous human c-*myc* locus and related pseudogenes may help to elucidate the origin and relationship of these various *myc*-related sequences.

The functional role of the *myc*-related sequences in the HCMV genome is unclear, as is the precise function of the v-*myc* or c-*myc* gene products. There is evidence, however, that the

p110$^{gag\text{-}myc}$ protein encoded by MC-29 virus is located in the nucleus and has DNA-binding properties (37, 38). *In vivo*, the avian retrovirus MC-29 can cause the proliferation and transformation of several types of target cells to form myeloid tumors, renal and hepatic carcinomas, and sarcomas; *in vitro* it can transform both macrophages and fibroblasts (39). The endogenous c-*myc* gene has also been implicated in the induction of B-cell lymphomas in chickens by avian leukosis virus (40–44). The c-*myc* locus appears to be activated by the integration of the avian leukosis virus long terminal repeat in the vicinity of the c-*myc* gene but the exact mechanism is unclear. The c-*myc* gene may also play a role in the genesis of a human tumor, Burkitt lymphoma, which is associated with the herpesvirus Epstein–Barr virus. Recent evidence indicates that the characteristic $8q^{-}14q^{+}$ translocation found in Burkitt lymphoma cells involves moving the endogenous human c-*myc* gene on chromosome 8 to the immunoglobulin heavy chain coding region on chromosome 14 (reviewed in ref. 45). The pathogenesis of these lymphomas suggests a multistep process involving more than a single transformation event. In the case of the chicken bursal lymphomas, although c-*myc* expression is increased a different transforming gene is detected by transfection of the DNA from the bursal lymphomas into NIH 3T3 cells (46). Thus, c-*myc* gene activation may be an early step followed by activation of another transforming gene. This latter point may be relevant to the finding that the transforming region of HCMV as detected by DNA transfection (6) does not contain the HCMV *myc*-related sequences.

HCMV is capable of multiple pathogenic manifestations in the human host. What role, if any, the *myc*-related sequences play in the onset of HCMV mononucleosis, birth defects, or cancers such as Kaposi sarcoma remains to be determined. Further consideration of this issue would be greatly facilitated by knowledge of the function of the *myc* gene product in normal and in neoplastic cells.

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