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Interferon Prevents the Generation of Spontaneous Deletions at the Left Terminus of Vaccinia Virus DNA

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In this report we have shown that Friend erythroleukemia cells persistently infected with vaccinia virus maintain the persistent infection even after 1 year of continuous interferon (IFN) treatment. The persistently infected cultures were responsive to IFN as determined by their ability to induce 2-5A synthetase, to increase the intracellular levels of 2-5A, and to cause rRNA cleavage. While large deletions at the left terminus of vaccinia DNA occurred readily in the virus population from untreated cells, IFN completely suppressed the generation of these spontaneous deletions. Removal of IFN from these cultures led to the appearance of similar deletions at the left terminus of the viral genome. The regions deleted contain more than half of the left-end inverted terminal repetition of the vaccinia genome. These findings show that IFN alters specific events associated with the generation of vaccinia DNA deletions.

Interferons (IFN) exert many biological effects on animal cells. They are antiviral, inhibit tumor cell growth, and have a regulatory role in the immune system (33). A number of RNAs and proteins are induced in the response of cells to IFN (13, 36), and these proteins either alone or in combination are thought to play a role in the antiviral and antiproliferative activity of IFN (3, 16). Some of the newly synthesized proteins are inactive, however, until the cells are infected by a virus or exposed to double-stranded RNA (3). Although the proteins induced by IFN, i.e., 2-5A synthetase, endoribonuclease, and eucaryotic initiation factor 2 protein kinase, have been generally considered likely mediators of the inhibition of viral protein synthesis, based on experiments conducted with cell-free systems, there is no direct correlation between their inducibility and antiviral functions. The molecular mechanisms involved in the decrease of cellular growth rates by IFN remain almost completely unknown (35). Attempts to correlate this inhibition with activation of the 2-5A synthetase-endoribonuclease and protein kinase systems have not been successful, although the possibility exists that very low levels of 2-5A or alternative products of the synthetase could be involved (32). Similarly, the reduction in thymidine uptake and phosphorylation that occurs in some IFN-treated uninfected cells does not correlate with inhibition of DNA synthesis (14). Accumulation of cells in the G_1-G_0 phase of the cell cycle, a characteristic of IFN-treated cells (35), might then be caused by some other phenomenon.

Studies on the mechanism of the antiviral action of IFN on vaccinia virus revealed that IFN can inhibit virus replication in some cells (11) and not in others (23, 31). The ability of vaccinia virus to escape blockade by the IFN system is thought to be mediated by viral gene functions (23, 31, 37, 38). The vaccinia virus particles from IFN-treated infected cells are infectious, but they have protein modifications and reduced adsorption to cells (9). Since vaccinia is a cytocidal virus, failure of IFN to inhibit virus replication causes, in turn, extensive cell damage, and the cells die after a few rounds of virus multiplication. The intracellular accumulation of large amounts of 2-5A and of viral double-stranded RNA late in infection might play a role in cell damage (23, 31; Paez and Esteban, submitted for publication). The characteristic wide host range of cells for vaccinia virus replication and the killing effect that occurs in both permissive and nonpermissive cells do not permit an evaluation of the efficacy of IFN in long-term cultures of vaccinia virus-infected cells. The establishment of cell lines persistently infected with vaccinia virus (30) offered an opportunity to establish whether IFN can "cure" infected cells and provided the means to characterize further the mode of action of IFN on this 122-megadalton (MDa) DNA-containing virus that multiplies in the cytoplasm of infected cells and encodes many of the enzymes necessary for viral DNA and RNA biogenesis (5). In this report we have characterized the effect of continuous IFN treatment on the replication of vaccinia virus in persistently infected Friend erythroleukemia (FEL) cells. Our results show that even after 1 year of continuous IFN treatment, FEL cells maintain the persistent virus infection. However, while in untreated cells there are deletions at the left terminus of the viral genome in all virus isolates, these deletions are completely prevented by IFN.

MATERIALS AND METHODS

Cells and viruses. FEL cells (a subclone of line 745A) were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum and antibiotics. This kind of cell has previously been used to study antiviral and IFN-mediated enzyme activities during infection with vaccinia virus (23). The plaque-purified WR strain of vaccinia virus was grown in HeLa S_3 Spinner cells at a multiplicity of 0.1 PFU per cell and was purified essentially according to the method of Joklik (15). The particle-to-PFU ratio was about 27 when the cells were titrated on monolayer cultures of monkey BSC-40 cells. The stock of virus used represents one single plaque isolate of vaccinia virus that was grown on a 150-mm plate and amplified in 3×10^9 HeLa S_3 cells. The initial vaccinia virus was obtained from R. Bablanian at Downstate Medical Center and has been maintained in cell culture for over 10 years. vP142 is a mutant of vaccinia virus with a 6.3-MDa deletion mapping at about 6.8 MDa from the left terminus (26). This mutant also contains an intact insert of the hemagglutinin gene of influenza virus. vP142 was kindly provided by E. Paoletti of the New York State Department of Health, Albany.

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IFN. Virus-induced mouse fibroblastoid IFN (containing 15% α and 85% β species) was prepared and partially purified on an antibody affinity column at a specific activity of 6×10^7 U of protein per mg. Titrations were carried out in mouse L-929 cells with vesicular stomatitis virus (VSV) as a challenge virus against a mouse IFN standard (G002-90511) from the Antiviral Substances Program, National Institutes of Health. IFN concentrations are given in reference units.

2-5A synthetase, 2-5A, and rRNA cleavage. The levels of 2-5A synthetase activity in cell extracts (S10) were determined after measuring the incorporation of [^{3}H]ATP into pppA2′p5′A or longer chains. The enzyme in S10 cell extracts (75 μl, equivalent to 2×10^6 cells) was bound to poly(I)-(C) agarose beads (50 μl of a 50% [vol/vol] suspension) and then washed, and the partially purified poly(I)-(C) enzyme was incubated with ATP. The product 2-5A was subsequently applied and eluted from DEAE-cellulose, and samples were assayed for radioactivity and by polyethyleneimine-cellulose chromatography (23). A radiobinding assay was used to measure the intracellular levels of 2-5A (31). Trichloroacetic acid (TCA)-soluble extracts were obtained from about 20×10^6 cells after the cells were suspended in 0.5 ml of 10% TCA. The supernatant was collected, extracted once with a 0.5-ml mixture of Freon–tri-*n*-octylamine (3:1, vol/vol), vortexed, and spun for 1 min in a Microfuge. The upper phase was lyophilized and suspended in half of the packed-cell volume of buffer A (20 mM Tris hydrochloride [pH 7.6], 85 mM potassium chloride, 5 mM magnesium acetate, 1 mM ATP, 5% glycerol). Each radiobinding assay (50 μl) contained 10 μl of TCA-soluble extract (7×10^6 cell equivalents), 20 μl of rabbit reticulocyte lysate, 10 μl of ppp(A2′p)$_3$A(^{32}P)pCp (3,000 Ci/mmol, Amersham Corp.) at 4 nCi/10 μl in buffer A, and 10 μl of distilled water. A calibration curve was run in parallel with 2-5A tetramer 5′-triphosphate at various concentrations (0.1 to 5 nM). The assays were incubated at 4°C for 90 min, and the protein-bound 2-5A was measured by filtration through nitrocellulose filters (HAWP-024, Millipore Corp.) presoaked with buffer B (buffer A minus ATP), washed three times with 1 ml of buffer B per wash, dried, and counted for radioactivity in toluene-based scintillant. RNA was isolated from cell pellets (50×10^6 cells). The isolation, denaturation, and electrophoresis of RNA in 1.8% agarose gels and staining were carried out as previously described (11).

Characterization of intracellular virion DNAs. Intracellular DNA was prepared from cell lysates (20×10^6 to 50×10^6 cells) during different passages. Cells were collected by centrifugation, suspended in 10 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–10 mM EDTA (2×10^7 cells per ml), lysed with 0.5% sodium dodecyl sulfate, digested with 50 μg of proteinase K per ml (1 h, 37°C), incubated overnight at 37°C with rotation, and extracted once with an equal volume of phenol (saturated in 0.1 M Tris hydrochloride [pH 7.9]) and twice with phenol-chloroform-isoamylalcohol (50:48:2, vol/vol). To the aqueous phase, 2.5 volumes of absolute ethanol was added in the presence of 0.4 M NaCl, and the DNA was immediately collected with a glass rod, drained, dried, and suspended in 10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA at a final concentration of about 0.5 mg/ml. Conditions for isolation of WR DNA from purified virus, digestion of DNA with restriction endonucleases, gel electrophoresis, and Southern blotting and hybridization with specific nick-translated DNA probes have been previously described (4, 23, 27). Virus from persistently infected cells was plaque purified by selection of well-isolated plaques on monolayers of BSC-40 cells. Independent virus isolates were propagated in BSC-40 cells, and virus was purified as previously described (9). When appropriate, these viruses were labeled in the DNA with [^{3}H]thymidine (9).

RESULTS

IFN does not prevent the establishment of a persistent infection in FEL cells infected with vaccinia virus. It has previously been shown that persistent infection with vaccinia virus can be established in cultures of murine FEL cells (30). This system offers the opportunity to establish whether IFN can cure cells persistently infected with vaccinia virus and provide the means to measure the level(s) of antiviral action. FEL cells were infected with purified vaccinia virus at 1.0 PFU per cell. On days 2 and 3 of infection, >90% of the cells had died and remained attached to the dish. Surviving cells were removed by centrifugation and suspended in fresh medium. After recovery (2 to 3 weeks), the cells were serially passaged every 4 to 5 days. At passage 7, half of the cultures were treated with 500 U of mouse IFN per ml, and this treatment was maintained continuously during changes of medium every 4 to 5 days. Cells maintained continuously in culture for short and long passages were titrated for virus production in BSC-40 cells by plaque assay. Table 1 shows the yields of infectious vaccinia virus from persistently infected FEL cells in the absence or presence of IFN. In untreated infected FEL cells, virus yields occurred in cycles, and when the virus yield reached titers similar to those of lytic infection of FEL cells with wild-type virus (20 PFU per cell), many cells lysed, but the surviving cells proliferated and initiated another cycle of virus production. In IFN-treated infected FEL cells, virus yields also occurred in cycles. IFN caused a slight decrease in virus yields, which in turn reduced the effect of cell lysis. Significantly, when the virus plaque size was compared between persistently infected cultures with or without IFN treatment, a clear distinction was made (Fig. 1A). While in

TABLE 1. Effect of IFN on virus yields in FEL cells persistently infected with vaccinia virus[a]

Passage no.	Virus yield (PFU/cell) with IFN concn (U/ml) of:	
	0	500
7	0.4	0.4
8	2.3	1.0
9	16.6	6.3
14	1.4	7.65
25	11.6	0.35
28	6.3	10.4
30	18.0	12.5
31	1.3	1.5
40	0.03	2.6
42	1.3	3.0
48	8.0	12.5
70	1.75	0.52
77	0.8	1.0

[a] FEL cells were infected with 1 PFU of vaccinia virus per cell, and surviving cells were serially passaged every 4 to 5 days. At passage 7, half of the cultures were treated with IFN (500 U/ml), and this treatment was maintained during serial passages. Samples were taken at various passages, and the intracellular virus yields were estimated in BSC-40 cells by plaque assay. In untreated and IFN-treated nonpersistent FEL cells infected with 5 PFU of wild-type vaccinia virus per cell and collected at 24 h postinfection, virus yield was 22.0 and 18.0 PFU per cell with IFN at 0 and 500 U/ml, respectively.

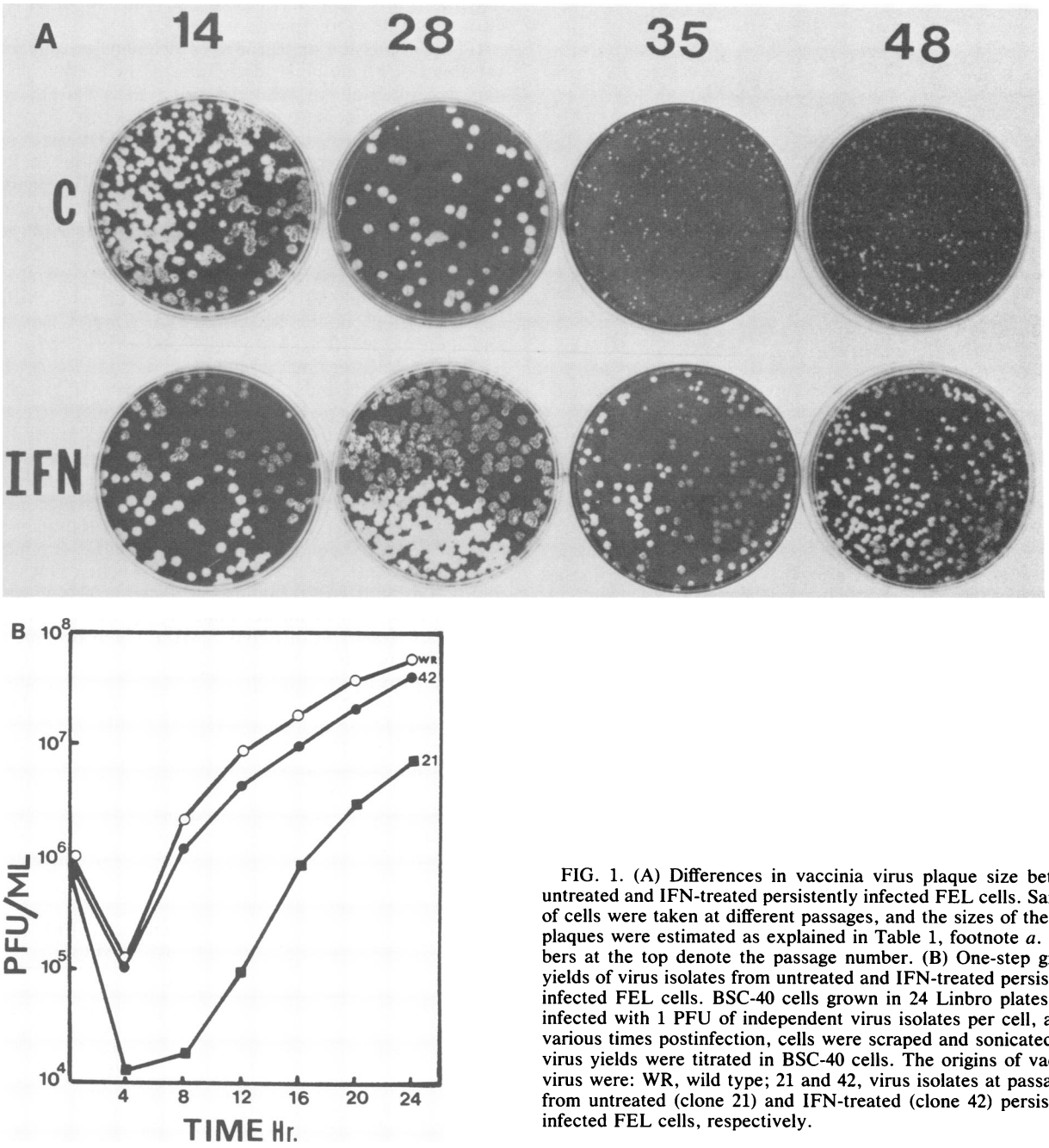

FIG. 1. (A) Differences in vaccinia virus plaque size between untreated and IFN-treated persistently infected FEL cells. Samples of cells were taken at different passages, and the sizes of the virus plaques were estimated as explained in Table 1, footnote *a*. Numbers at the top denote the passage number. (B) One-step growth yields of virus isolates from untreated and IFN-treated persistently infected FEL cells. BSC-40 cells grown in 24 Linbro plates were infected with 1 PFU of independent virus isolates per cell, and at various times postinfection, cells were scraped and sonicated, and virus yields were titrated in BSC-40 cells. The origins of vaccinia virus were: WR, wild type; 21 and 42, virus isolates at passage 48 from untreated (clone 21) and IFN-treated (clone 42) persistently infected FEL cells, respectively.

untreated cultures the plaque size was dramatically reduced with long passages, in IFN-treated cultures the plaque size was only slightly reduced. The fact that the virus plaque size is reduced with prolonged passages implies that the virus population in untreated cultures has lost infectivity, while in IFN-treated cultures the virus retains its infectivity. This result was documented after measuring one-step growth yields of independent virus isolates from untreated and IFN-treated persistently infected FEL cells (Fig. 1B).

IFN-mediated antiviral activities in FEL cells persistently infected with vaccinia virus. Failure of IFN to cure FEL cells persistently infected with vaccinia virus could be attributed to modifications in the response of these cells to IFN. Therefore, to document if FEL cells were responsive to IFN treatment, we measured various parameters that have been correlated with antiviral action of IFN (3, 16). These parameters include inhibition of viral protein synthesis and activation of the 2-5A synthetase-endoribonuclease system. The synthesis of VSV proteins was completely blocked by IFN in nonpersistent FEL cells (Fig. 2A). In cultures of FEL cells persistently infected with vaccinia virus, the synthesis of viral proteins was not blocked by IFN (Fig. 2B). This result was also confirmed after immunoprecipitation of the labeled proteins with rabbit antivaccinia antiserum followed by sodium dodecyl sulfate-polyacrylamide electrophoresis analysis (data not shown). When persistently infected FEL cells were superinfected with VSV, IFN treatment did not lead to inhibition of VSV protein synthesis (Fig. 2B). These

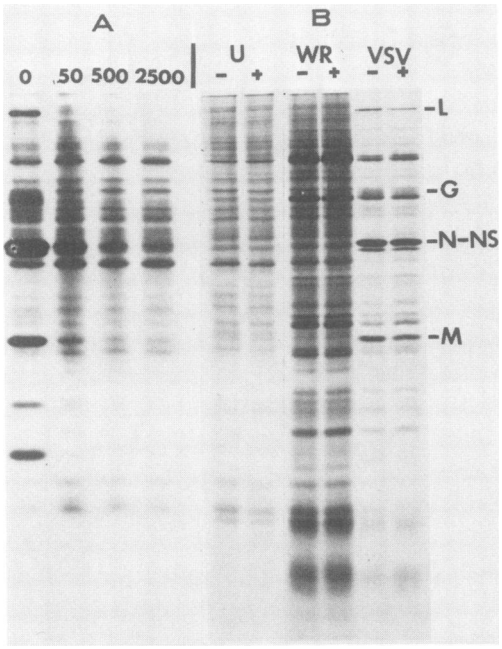

FIG. 2. Response of FEL cells to IFN as measured by the extent of protein synthesis. Polyacrylamide gel electrophoretic analysis of the ^{35}S-labeled proteins synthesized in untreated and IFN-treated FEL cells persistently infected with VSV (A) and with vaccinia virus (B). For panel A, cells were infected with 5 PFU of VSV per cell and labeled for 1 h with 20 μCi of [^{35}S]methionine per ml at 5 h postinfection. Concentrations (in units per milliliter) of IFN used to treat the FEL cells are given at the top. For panel B, cultures of persistently infected FEL cells in passage 10 were split into halves. One half was labeled directly, and the other half was first infected with VSV (5 PFU per cell) and then labeled with [^{35}S]methionine at 5 to 6 h postinfection. Proteins were analyzed in 12% gel as previously described (23). −, untreated cells; +, IFN-treated (500 U/ml) cells; U, uninfected cells; WR, cells persistently infected with vaccinia virus; VSV, cells persistently infected with vaccinia virus and superinfected with VSV. The migrations of VSV proteins L, G, N-NS, and M are indicated.

findings are in agreement with previous observations in doubly infected cells (23, 37) and provide further evidence with a different system that vaccinia virus is resistant to IFN and that while IFN normally blocks VSV protein synthesis, vaccinia prevents this inhibition from occurring. The basis for vaccinia virus resistance to IFN is not known, but it may be related to viral specific products that can dephosphorylate 2-5A (23, 24; Paez and Esteban, submitted) and inhibit the protein kinase system (23, 30a, 37, 38). Activation of the 2-5A synthetase-endoribonuclease system was determined after the measurement of levels of 2-5A synthetase and intracellular accumulation of 2-5A and the extent of cleavage of rRNA during the course of the persistent infection. 2-5A synthetase was activated by IFN in nonpersistent and persistently infected FEL cells (Table 2). The variations found in the levels of 2-5A synthetase activity in infected cells are most likely related to the degree of virus infection of cells (23). When the intracellular levels of 2-5A were measured in TCA-soluble extracts by radiobinding assay (31), we found in IFN-treated persistently infected cells levels of 2-5A higher than in untreated persistently infected cells. The 2-5A products are most likely the activators of the 2-5A-dependent endoribonuclease, as measured from the extent of rRNA cleavage. We conclude that FEL cells persistently infected with vaccinia virus are responsive to IFN even during long-term treatments.

IFN prevents the appearance of deletions on intracellular vaccinia DNA from persistently infected FEL cells. The findings described above establish that FEL cells persistently infected with vaccinia virus are responsive to IFN but that IFN did not "cure" these cells even after 1 year of continuous treatment. Since genetic changes (additions, deletions, and rearrangements) occur readily during serial passage of vaccinia virus (19, 20, 26), it was of interest to determine if in persistently infected cells IFN could alter viral genetic changes. Therefore, we examined the state of intracellular vaccinia DNA. DNA was extracted from cultures of persistently infected cells at various passages, digested with various restriction endonucleases (*Eco*RI, *Hin*dIII, and *Xho*I), and then blotted (Fig. 3A and B) and hybridized with the 6.3-MDa *Eco*RI terminal fragment of vaccinia DNA which is repeated at both ends (39). As expected, viral sequences were found during all passages, regardless of IFN treatment. However, clear differences in the sizes of the viral DNA fragments were observed between cultures. In untreated infected FEL cells, a major 8-MDa deletion was detected by analysis of the *Hin*dIII digest (Fig. 3A). The 8-MDa deletion was observed at early passages and became dominant at late passages. This deletion occurred in *Hin*dIII fragment C, which represents the left terminus of vaccinia DNA, while the right *Hin*dIII-B terminus of vaccinia DNA was not altered. Digestion of intracellular DNA with *Eco*RI generated a new fragment of about 5 MDa which became dominant at late passages (Fig. 3B, lanes 2 and 4). Our strain of vaccinia virus has an extra *Eco*RI site at each terminus, thus generating fragments of about 4 and 2.1 MDa, of which the former was found at both ends (Fig. 3B, lane 1). A third minor fragment of about 3.5 MDa was also present. Control experiments were carried out to show that the 3.5-MDa fragment was caused by a mutant with a small deletion in our stock of purified virus. Ten independent plaque-purified virus isolates were used to infect monkey BSC-40 cells, and intracellular DNA was analyzed after *Eco*RI digestion by

TABLE 2. IFN-mediated enzyme activities in FEL cells persistently infected with vaccinia virus[a]

Type of infection (passage no.) and treatment	Activity					
	2-5A synthetase (cpm/μg) at IFN concn (U/ml) of:		Intracellular 2-5A (nM) at IFN concn (U/ml) of:		rRNA cleavage at IFN concn (U/ml) of:	
	0	500	0	500	0	500
Nonpersistent (0)						
U	155.7	480.7	<1.0	<1.0	−	−
I	97.3	110.5 (77)	2.5	25.0	−	++
Persistent (14)						
FEL-0	125.2	230.7 (52)	<1.0	3.5	−	+
FEL-500		168.2 (65)		10.0		++
Persistent (25)						
FEL-0	102.4	145.5 (70)	<1.0	4.75	−	+
FEL-500		433.0 (10)		<1.0		−

[a] Levels of 2-5A synthetase, intracellular accumulation of 2-5A, and rRNA cleavage were measured in FEL uninfected (U), infected (I; 100 EB per cell for 8 h postinfection), and persistently infected cells without (FEL-0) or with (FEL-500) continuous IFN treatment as described in Materials and Methods. Numbers in parentheses are percentages of inhibition relative to levels found in uninfected cells. The results for rRNA cleavage are given in a semiquantitative form. −, No cleavage; +, 25% cleavage; ++, 50% cleavage.

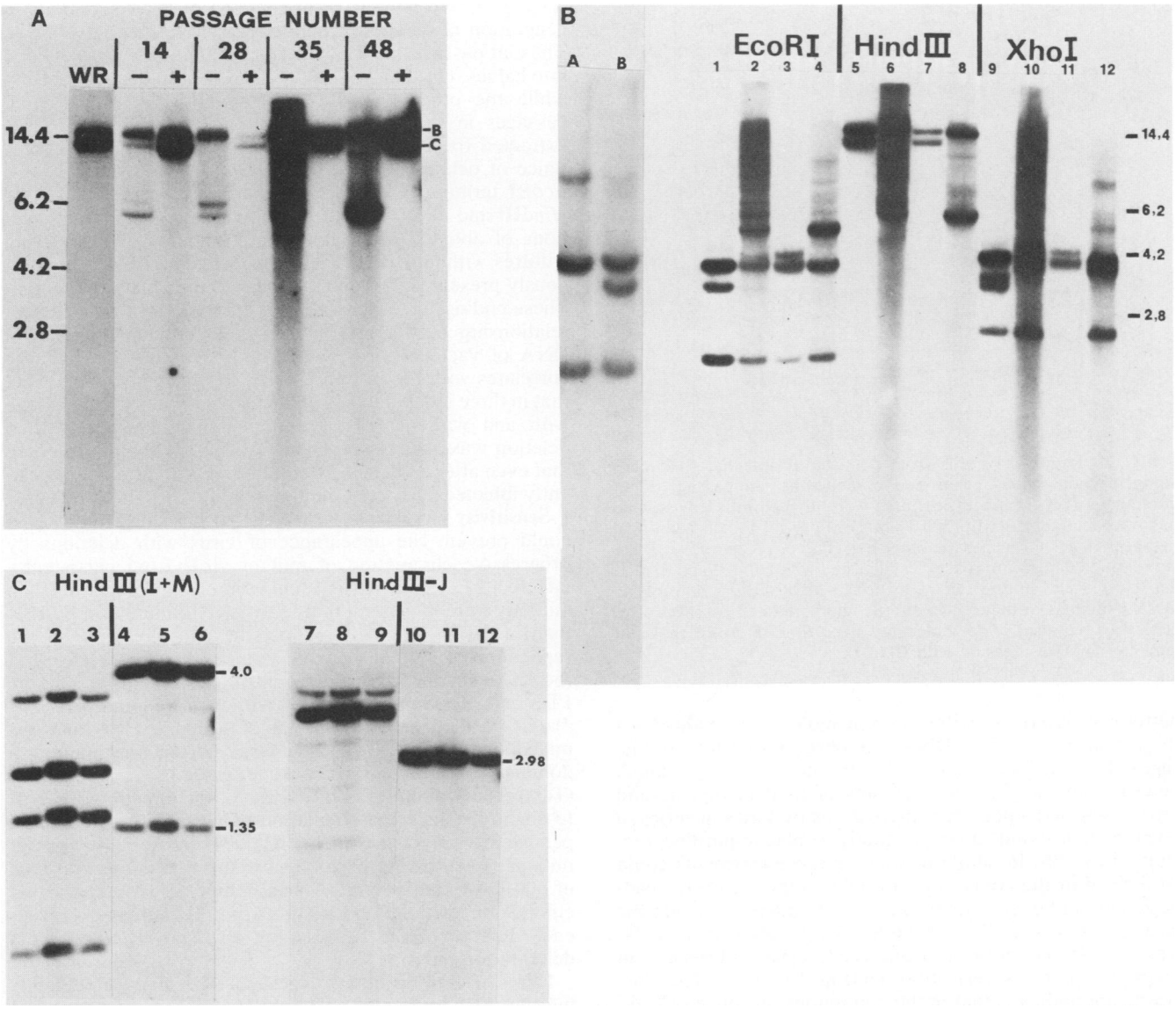

FIG. 3. State of intracellular vaccinia DNA in untreated and IFN-treated persistently infected FEL cells. At passages 14, 28, 35, and 48, high-molecular-weight DNA was extracted from whole-cell lysates, digested with *Eco*RI, *Hin*dIII, and *Xho*I, run on 0.7% agarose gel, and blotted onto nitrocellulose paper. (A) Intracellular DNA extracted at different passages and digested with *Hin*dIII. (B) Intracellular DNA extracted at passages 35 and 48 and digested with *Eco*RI, *Hin*dIII, and *Xho*I. In panels A and B, DNA was hybridized with ^{32}P-labeled nick-translated 6.3-MDa termini of vaccinia DNA cloned in phage lambda and kindly provided by B. Moss, National Institutes of Health. The origins of the DNAs of panel A are vaccinia virus (WR) and untreated (−) and IFN-treated (+) persistently infected cells at passages 14, 28, 35, and 48. The origins of the DNAs for panel B are as follows: vaccinia virus (lanes 1, 5, and 9), untreated persistently infected cells at passage 35 (lanes 2, 6, and 10), persistently infected cells at passage 48, both IFN treated (lanes 3, 7, and 11) and untreated (lanes 4, 8, and 12). The molecular sizes (in MDa) of lambda DNA digested with *Hin*dIII are indicated. To the left in panel B is a blot of *Eco*RI digest of intracellular DNA from BSC-40 cells infected with plaque-purified isolates from the two populations of vaccinia virus that were present in our original stock. Cells were infected with 1 PFU per cell, and total DNA was collected at 24 h postinfection. Patterns are of intracellular DNA from cells infected with wild-type virus (lane A) and with vaccinia virus but with a 0.5-MDa deletion (lane B). The band of about 8 MDa is most likely the result of head-head and tail-tail concatemers (21). In panel C, DNA extracted at passage 48 and digested with *Eco*RI (lanes 1 through 3 and 7 through 9) and *Hin*dIII (lanes 4 through 6 and 10 through 12) was hybridized with vaccinia DNA *Hin*dIII fragment I plus M or J that was used as a probe. The origins of DNAs were vaccinia (lanes 1, 4, 7, and 10) and untreated (lanes 2, 5, 8, and 11) and IFN-treated (lanes 3, 6, 9, and 12) persistently infected FEL cells. The molecular sizes (in MDa) of vaccinia *Hin*dIII fragments I, J, and M are given.

Southern blots. The pattern of one of the virus isolates (Fig. 3B, lane A) was that of the wild type (representing 80% of the virus population), while the pattern of the other virus isolate (lane B) corresponded to a virus with a 0.5-MDa deletion (20% of the virus population). This deletion maps within the terminal *Sal*I fragment (not shown). Digestion of wild-type vaccinia DNA with *Xho*I generated a major fragment of about 4.1 MDa, which represented the two identical ends (Fig. 3B, lane 9) (6). With intracellular DNA digested with *Xho*I, a prominent doublet of 4.1 and 3.8 MDa was observed, the latter part formed as a result of the deletion on the left terminus (Fig. 3B, lanes 10 and 12). A heterogeneous

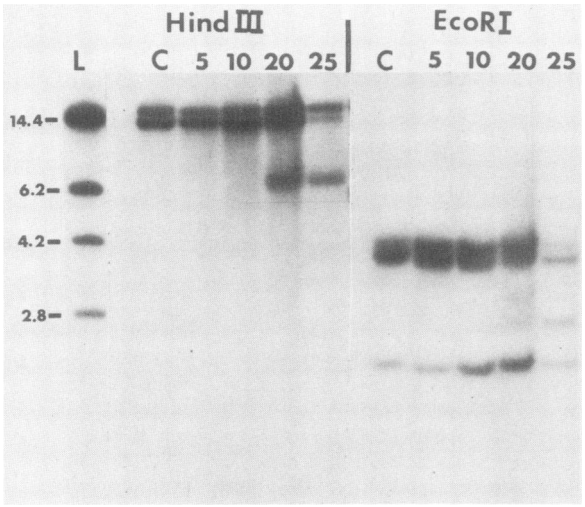

FIG. 4. Removal of IFN from persistently infected FEL cells leads to spontaneous generation of deletions. At passage 30 of continuous IFN treatment, cultures were divided into halves. One half was maintained with 500 U of IFN per ml for 25 additional passages (lanes C), while the other half did not receive IFN. DNA was extracted at various passages, digested with *Hin*dIII and *Eco*RI, and blotted and hybridized with the 6.3-MDa termini of vaccinia DNA. Numbers at the top denote the number of cell passages after IFN was removed. The molecular sizes (in MDa) are those of lambda (L) DNA digested with *Hin*dIII.

pattern of fragments with different molecular weights than the termini of vaccinia DNA was observed during all passages (Fig. 3B, lanes 2, 4, 6, 8, 10, and 12). This terminal heterogeneity might represent sets of tandem repeats and intervening sequences that alternate many times in series, a phenomenon found to occur readily in plaque-purified vaccinia virus (20). In addition, some of these fragments could be formed in the course of viral DNA replication by head-head and tail-tail concatemers (21) or by integration with the host chromosome (27) or both. When DNA from IFN-treated infected cells was analyzed, clear differences in restriction patterns were observed (Fig. 3A and B). The most significant finding is that in the continuous presence of IFN, the generation of deletions as well as the appearance of terminal heterogeneity was prevented, as was shown with *Hin*dIII (Fig. 3A and B, lane 7). However, some DNA alterations were observed in both the *Eco*RI and *Xho*I digests (Fig. 3B, lanes 3 and 11) compared with parental virus. The disappearance of the band corresponding to virus with a small deletion may be the result of selection of wild-type over mutant virus by IFN. It took 28 passages for this band to disappear in IFN-treated infected FEL cells (data not shown). The appearance of a doublet at each terminus (Fig. 3B, lanes 3 and 11) was probably due to variations in the number of tandem repeats in the mixed virus population from IFN-treated persistently infected FEL cells (20, 40).

To establish if structural alterations of intracellular vaccinia DNA are also produced in other regions of the viral genome, Southern blots were hybridized with internal *Hin*dIII fragments I, J, and M (6). The results (Fig. 3C) revealed no changes in the internal structure of intracellular vaccinia DNA in persistently infected FEL cells with or without IFN treatment.

Since IFN was maintained in cultures continuously, it was critical to determine if, after removal of IFN, spontaneous generation of deletions could appear in intracellular DNA. Thus, at passage 30 of IFN treatment, cultures were divided into halves. One half was maintained continuously with IFN, while the other half did not receive IFN. At different passages in the absence or presence of IFN, DNA was extracted from these cultures and analyzed for the occurrence of deletions by blot hybridization with the 6.3-MDa *Eco*RI termini of vaccinia DNA. The results (Fig. 4) for *Hin*dIII and *Eco*RI digestions show the appearance of deletions of about 7 MDa at the left terminus in DNA from cultures without the IFN regimen, while if IFN was continuously present, DNA deletions were completely prevented. These findings provide additional evidence for a cause-effect relationship between IFN and prevention of deletions in DNA of vaccinia virus. That the absence of the deletion correlates with the presence of IFN is indicated by the fact that in three independent established cultures during passage with and without IFN, the appearance of virus with a deletion was always prevented by IFN. It should be noted that even after 2 years of continuous IFN treatment, persistently infected FEL cells did not exhibit virus with deletions.

Sensitivity of vaccinia virus deletion mutants to IFN. IFN could prevent the appearance of virus with deletions by progressive elimination of mutant virus from persistently infected FEL cells. This elimination could occur if such mutants were in general more sensitive to inhibition by IFN than are wild-type virus. Thus, the effect of IFN on the replication of mutant vaccinia virus containing deletions at the left terminus was studied in different systems, i.e., in FEL cells persistently infected with (i) a well-characterized plaque-purified deletion mutant of vaccinia virus or (ii) a mixed population of vaccinia virus particles containing a dominant deletion of 8 MDa. As assessed by virus titrations (Table 3) and Southern blots (Fig. 5), the effect of IFN was to slightly reduce the population of virus with deletions in persistently infected cultures. Cure of these cells was not obtained even during 40 passages in the continuous presence of 500 U of IFN per ml. These findings clearly show that elimination of deletion mutants from IFN-treated persistently infected FEL cells is not the way IFN prevents deletion formation.

Effect of IFN on mature vaccinia DNA from persistently infected FEL cells and physical map of the deletions. The findings described above demonstrate that IFN prevents the occurrence of deletions at the left terminus of vaccinia DNA during virus replication in persistently infected FEL cells and that elimination of deletion mutants is not the way IFN inhibits deletion formation. Since these results were obtained with intracellular DNA which represents a heterogeneous population of DNA molecules, we next determined if the mature DNA assembled into virions was altered by IFN in persistently infected cultures, and we also established the physical map of the deletions. Thus, plaque-purified virus was isolated at passage 48 and grown, and DNA was extracted and then analyzed by Southern blots after hybridization with specific probes. Blots of *Hin*dIII, *Xho*I, *Kpn*I, and *Eco*RI digests were hybridized with the 6.3-MDa terminal fragment of vaccinia DNA (Fig. 6). As observed in the *Hin*dIII digest (Fig. 6A), the left (frament C) and right (fragment B) termini of vaccinia DNA were maintained in DNA of virus isolates from IFN-treated cells (clones 31, 42, and 47), while the left end was deleted in DNA of virus isolates from untreated cells (clones 3, 7, and 21). The left-end deletion generated with *Hin*dIII a new fragment of about 5.8 MDa. With *Xho*I digests (Fig. 6A), the 4.1-MDa

right and left termini of vaccinia DNA, which comigrated in wild-type viral DNA (fragments H and I) (6), were also maintained in DNA of virus isolates from IFN-treated cells. Small differences in migration of the 4.1-MDa fragment were noticeable between clones. With DNA of virus isolates from untreated cells, the deletion generated a new fragment of about 3.8 MDa in some clones, while in other clones the fusion fragment comigrated with the right terminus. With *Kpn*I (Fig. 6A), the right and left termini of vaccinia DNA comigrated (fragments J and K) (6), while the deletion generated a new fragment of about 9.6 MDa which was not present in DNA of virus isolates from IFN-treated cells. As with the *Xho*I digest, small differences in the migration of the 3.2-MDa fragment were observed between clones. With *Eco*RI digests (Fig. 6B), the 4-MDa fragment corresponding to both ends of vaccinia DNA was preserved in DNA of virus isolates from IFN-treated cultures (clones 42 and 47). The left-end deletion generated with *Eco*RI a new fragment of about 5 MDa. Small differences in migration of the 4-MDa fragment were also observed between clones. Densitometric tracing of the uniformly ^{3}H-labeled virion DNA digested with *Eco*RI (Fig. 6B) revealed no evidence for transposition of sequences between the ends of the viral genome in DNA of virus isolates from untreated and IFN-treated cells (data not shown). The small differences in migration observed with the termini shown in Fig. 6 are probably due to changes in the number of tandem repeats (20, 40). By using a combination of restriction enzymes, we found that the physical map of the 8-MDa deletion started between 2.1 and 2.3 MDa from the left terminus of the viral genome (Fig. 6C). After removal of IFN from persistently infected cells, the 7-MDa deletion (Fig. 4) also mapped within the inverted terminal repetition starting between 2.3 and 2.6 MDa from the left end of the viral genome (Fig. 6C).

These findings establish that IFN prevents the occurrence of spontaneous deletions at the left terminus of vaccinia DNA from persistently infected cells. Small variations in the length of the tandem repeat region could be altered independently of IFN treatment.

TABLE 3. Effect of IFN on virus yields in FEL cells persistently infected with deletion mutants of vaccinia virus

Kind of cell[a] and passage no.	Virus yield (PFU/cell) with IFN concn (U/ml) of:	
	0	500
WR_{HA}		
4	0.01	0.01
15	0.24	0.1
20	7.0	2.5
25	3.3	3.3
40	5.6	2.5
WR_{Mix}		
80	0.8	0.8
85	0.04	0.02
100	9.6	6.4

[a] WR_{HA}; FEL cells were infected with 1 PFU per cell of purified vaccinia virus mutant (vP142) that contains a 6.3-MDa deletion at the left terminus (26) and an insert of intact hemagglutinin gene of influenza virus. Surviving cells were serially passaged as described in Materials and Methods. At passage 4, half of the cultures were treated with IFN (500 U/ml), and this treatment was maintained during serial passages. WR_{Mix}; persistently infected FEL cells containing a dominant 8-Mda deletion at the left terminus of vaccinia DNA were treated with IFN (500 U/ml) at passage 80, and this treatment was maintained throughout. Estimation of virus yields was performed as described in Table 1, footnote *a*.

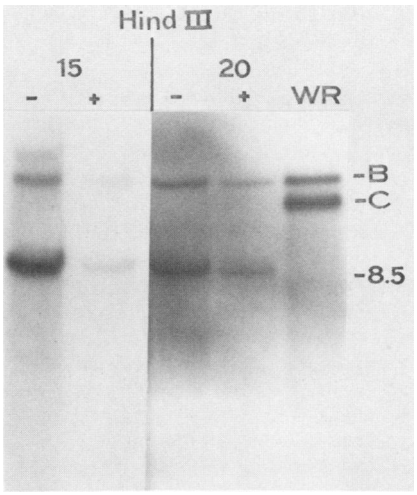

FIG. 5. Effect of IFN on the state of intracellular DNA from FEL cells persistently infected with a deletion mutant (WR_{HA}) of vaccinia virus. At passages 15 and 20, total DNA was extracted, digested with *Hin*dIII, and blotted and hybridized with the 6.3-MDa termini of vaccinia DNA. Symbols: −, untreated cells; +, IFN-treated cells; B and C, right and left termini of wild-type vaccinia DNA. The size of the deleted *Hin*dIII C fragment is indicated in MDa.

DISCUSSION

In this report we have shown that FEL cells persistently infected with vaccinia virus maintain the persistent infection even after 1 year of continuous IFN treatment. The yields of infectious vaccinia virus were slightly reduced in IFN-treated persistently infected cells. When virus yield reached 10 to 20 PFU per cell, many FEL cells lysed, and IFN treatment reduced this process (Table 1). The surviving cells proliferated and initiated another cycle of virus production. Under these conditions, the rate of growth of persistently infected cells compared with that of uninfected cells decreased, and IFN had little effect on cell growth during the persistent state. Whereas in untreated cultures the plaque size was dramatically reduced with passage number, in IFN-treated cultures the plaque size was only slightly reduced (Fig. 1A). This result implies that the virus population in untreated cultures had lost infectivity, while in IFN-treated cultures the virus retained its infectivity, as documented with one-step growth yields (Fig. 1B). These persistently infected cultures were responsive to IFN, as determined by their ability to induce 2-5A synthetase, to increase the intracellular levels of 2-5A, and to cause rRNA cleavage (Table 2). Southern blot hybridization analysis with total intracellular DNA digested with various restriction endonucleases and probed with the termini and internal fragments of vaccinia DNA revealed two major findings. First, whereas in untreated cultures large deletions occurred spontaneously at the left terminus of vaccinia DNA, the continuous presence of IFN in persistently infected cultures completely suppressed the occurrence of these deletions (Fig. 3), and second, removal of IFN from these cultures led to the appearance of similar deletions at the left terminus of vaccinia DNA (Fig. 4). Southern blot analysis, but with DNA from plaque-purified virus, revealed similar deletions at the left terminus of vaccinia DNA in virus isolates from untreated persistently infected cells which were completely blocked in virus isolates from IFN-treated persistently in-

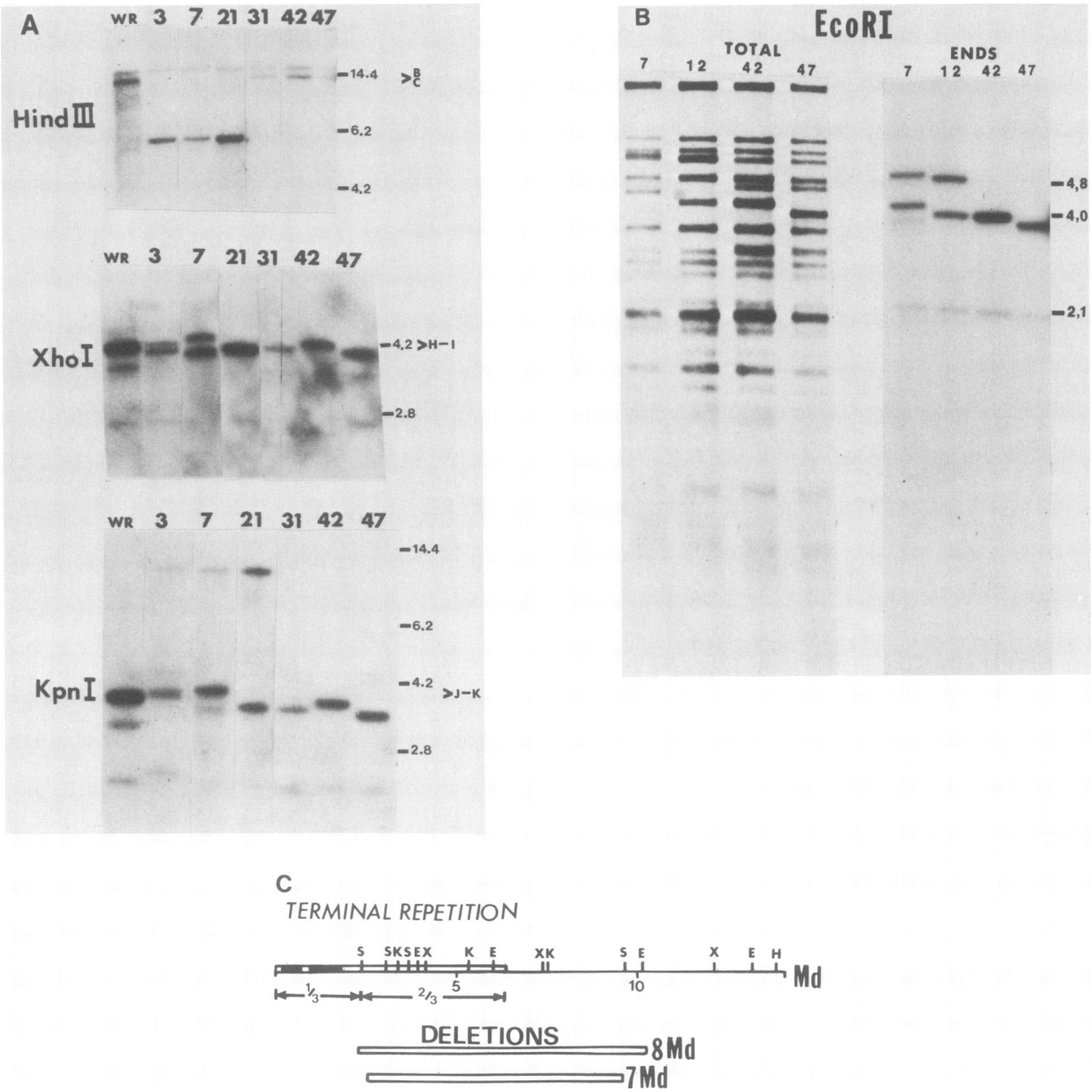

FIG. 6. Characterization of the termini of vaccinia virus DNA from untreated and IFN-treated persistently infected FEL cells. Blots of *Hin*dIII, *Xho*I, *Kpn*I (A), and *Eco*RI (B) digests of DNA isolated from plaque-purified virus that were hybridized with the 6.3-MDa termini of vaccinia DNA. (B) *Eco*RI digest of [^{3}H]thymidine-labeled viral DNA isolated from independent purified viruses. TOTAL, uniformly labeled viral DNA; ENDS, termini of vaccinia DNA. The molecular sizes (in MDa) are those of lambda DNA digested with *Hin*dIII (A) and vaccinia DNA termini digested with *Eco*RI (B). Numbers at the top denote the different clones of vaccinia virus obtained in passage 48 from untreated (clones 3, 7, 12, and 21) and IFN-treated (clones 31, 42, and 47) persistently infected FEL cells. (C) Restriction map of the left terminus of vaccinia DNA and localization of the characteristic 8- and 7-MDa deletions (Fig. 3 and 4, respectively) in virus isolates from persistently infected cells. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xho*I.

fected cells (Fig. 6). These findings suggest that IFN prevents the generation of deletions in persistently infected cells. However, some alterations were found in intracellular DNA from IFN-treated persistently infected cells (Fig. 3B). The fact that there is no apparent net loss in DNA mass in the presence of IFN does not preclude deletions of single-copy genes. It could be that deletion of unique sequences occurs in the presence of IFN but that replacement of the deleted material by other sequences follows. We have not observed encapsulation of incomplete forms of viral DNA in virions from untreated or IFN-treated persistently infected cells, nor have we observed reiteration of DNA sequences within the left terminus of vaccinia DNA or transposition of sequences between the ends.

IFN could prevent the generation of deletions at the left terminus of vaccinia DNA from persistently infected FEL cells by elimination of mutant viruses with selection of wild-type virus or by a direct effect on DNA processes or both. The first of these possibilities might play a limited role, since FEL cells persistently infected with deletion mutants

of vaccinia virus could not be cured even during 40 passages in the continuous presence of IFN (Table 3, Fig. 5). Moreover, generation of deletions occurred before the appearance of viruses with reduced infectivity (Fig. 1 and 3A). The fact that IFN selectively blocked the occurrence of deletions on vaccinia DNA strongly suggests that IFN has a direct effect on DNA-related processes. Several lines of evidence indicate that IFN alters specific DNA processes. First, from studies on the mode of replication of vaccinia virus, we have shown that IFN decreased the elongation of viral DNA, an effect which did not correlate with an inhibition of protein synthesis (10). Second, IFN inhibited marker rescue of vaccinia virus, an effect likely to be the result of the prevention of homologous recombination (25). Third, IFN inhibited genetic and oncogenic transformations by viral and cellular genes on NIH 3T3 and Ltk$^-$ cells (7, 28), and this inhibition appeared to occur at the level of stabilization or integration of exogenous DNA sequences in the recipient cell (28). Fourth, IFN inhibited formation of Okazaki fragments in some cell lines, and this inhibition has been related to turnover of DNA (18). The fact that IFN did not induce the degradation of mature vaccinia DNA, as observed in the course of virus infection (10), during marker rescue of vaccinia virus (25) or in persistently infected FEL cells (Fig. 3 through 6) strongly suggests that ligation-integration inhibitions could be a site of action for IFN. This inhibition could result from IFN-mediated changes in the topology of DNA, in the activity of DNA replication enzymes, or in factors involved in these DNA processes (12).

Generation of spontaneous deletions on vaccinia DNA could be due to recombinatory exchanges between direct repeats (1) or to illegitimate recombination (29) or to both. From studies with pneumococcal *mal* gene cloned in *Bacillus subtilis*, it has been suggested that a topoisomerase-like enzyme can produce deletions within repeated sequences (3 to 13 base pairs long), presumably by a recombination mechanism involving short homologies that serve as the recognition sites for the enzyme (17). Since vaccinia virus induces during infection a topoisomerase and this enzyme is encapsulated into virions (5), it is tempting to speculate that there are recognition sites for this enzyme at the left end of the viral genome which are not present on the right terminus. IFN might interfere with the activity of topoisomerase-like enzymes through changes in the topology of DNA, factor requirements, or protein modification. By the DNA sequence analysis of deletion junctions in mutant viruses, it should be possible to determine if short direct repeats mediate spontaneous deletions in DNA of vaccinia virus. Deletions or rearrangements near the ends of the viral DNA have been found in several poxviruses (2, 8, 19, 22, 26). The untreated and IFN-treated vaccinia virus FEL cell systems could provide the means to study genetic variability of vaccinia virus. These systems could also be useful in the study of the mechanism of deletion formation and its prevention by IFN, since deletions in vaccinia DNA can be readily seen after 10 passages (unpublished observations). These studies should be valuable in the investigation of the role played by somatic cell mutations in the process of carcinogenesis. In this regard, recombination between octanucleotide direct repeats within the chicken cellular gene homologous to the Rous sarcoma virus *src* gene has been implicated in the mechanism for generating the transforming virus (34). By altering specific DNA processes, such as blocking the generation of deletions and inhibiting ligation-integration, IFN provided a mechanism which might explain some of its own antitumor properties.

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