VIROLOGY 199, 491–496 (1994)

The Interferon-Induced Double-Stranded RNA-Activated Protein Kinase Induces Apoptosis

SEAN BONG LEE* AND MARIANO ESTEBAN*,†,‡,[1]

†Departments of Biochemistry and *Microbiology and Immunology, SUNY Health Science Center at Brooklyn, New York 11203; and ‡Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, 28049 Madrid, Spain

Received September 15, 1993; accepted December 7, 1993

Interferons (IFNs) exert antitumor activities, but the molecular mechanism underlying these effects is poorly understood. IFN-induced, double-stranded (ds) RNA-activated protein kinase (p68 kinase) has long been implicated in mediating the antiproliferative effects of IFN. In addition, recent studies suggest that p68 kinase may function as a tumor suppressor gene. In this investigation we showed that expression of p68 kinase in HeLa cells resulted in a rapid cell death characteristic of apoptosis. Rapid cell death was not observed in cells which expressed a mutant form of p68 kinase ($lys_{296} \rightarrow$ arg) indicating that cell death observed is the result of p68 kinase expression and activation. Moreover, infection of HeLa cells with the mutant vaccinia virus lacking E3L gene, which encodes a dsRNA binding protein that acts as an inhibitor of p68 kinase, also resulted in apoptosis. Thus, we propose that human p68 kinase functions as a tumor suppressor gene by actively participating in apoptosis.

Interferon (IFN)-induced protein kinase (p68 kinase) is a serine/threonine kinase which is autophosphorylated upon binding to double-stranded RNA (dsRNA) (*1, 2*). Autophosphorylation of p68 kinase leads to phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α), subsequently resulting in inhibition of translation initiation process (*1, 2*). Using vaccinia virus (VV) recombinants that either express a functional p68 kinase (WR68K) or a nonfunctional form ($lys_{296} \rightarrow$arg) of p68 kinase ($WR68K_{K \rightarrow R}$) both under the control of lacI repressor/operator controlling elements, we showed that viral and cellular protein synthesis were completely abolished at 24 hr postinfection (pi) in WR68K-infected cells in the presence of the inducer isopropyl-β-D-thiogalactoside (IPTG); this inhibition correlated with autophosphorylation of p68 kinase and was not observed in cells infected with $WR68K_{K \rightarrow R}$ (*3*). In the course of these studies, we noted extensive cell destruction in cells infected with WR68K but not in cells infected with $WR68K_{K \rightarrow R}$. Apoptosis, a process of programmed cell death characterized by cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation into nucleosome sizes (*4*), has been implicated in oncogenesis (*5*). Thus, we investigated the possibility that p68 kinase may function as a tumor suppressor gene and as a mediator of antitumor effects of IFN by inducing apoptosis.

As shown in Fig. 1, infection of HeLa cells with WR68K resulted in severe changes in cell morphology induced by expression of p68 kinase, which is distinct from that caused by $WR68K_{K \rightarrow R}$. In the presence of IPTG and at 48 hr pi, most of the cells infected with WR68K displayed a severe shrinkage together with small round structures characteristic of apoptotic bodies in the intercellular space (Fig. 1F). Most of the apoptotic cells were detached from the plate and were floating in the media. In the absence of IPTG, a subpopulation of cells also displayed cell shrinkage (Fig. 1E), and this is probably due to the leaky production of p68 kinase as previously described (*3*). $WR68K_{K \rightarrow R}$-infected cells did not show reduction in cell volume irrespective of IPTG treatment, indicating that the shrinkage of cells is due to expression and activation of a functional p68 kinase and not due to the virus (Figs. 1C–1D). Cell shrinkage was also not observed in cells infected with wild-type VV or in cells infected with another VV recombinant which expresses luciferase (data not shown). As determined by trypan blue exclusion test, <90% of cells died by 54 hr pi when the expression of p68 kinase was induced, whereas >60% of $WR68K_{K \rightarrow R}$-infected cells remained viable regardless of IPTG treatment (data not shown).

To visualize the effect of p68 kinase on the morphology of nuclei, cells were stained with fluorescent DNA binding dye Hoechst 33258 at 24 hr pi (Fig. 2). In cells infected with $WR68K_{K \rightarrow R}$ (Figs. 2C and 2D), nuclei appeared indistinguishable from those of mock-infected cells (Figs. 2A and 2B) except that viral DNA was also visible in the cytoplasm of infected cells. In WR68K-infected cells, however, shrinkage of nuclei, condensed chromatin around the periphery of the nuclear membrane, and segmentation of chromatin into small round bodies, characteristics of apoptotic nuclei (*4*), were

[1] To whom correspondence and reprint requests should be addressed.

0042-6822/94 $6.00

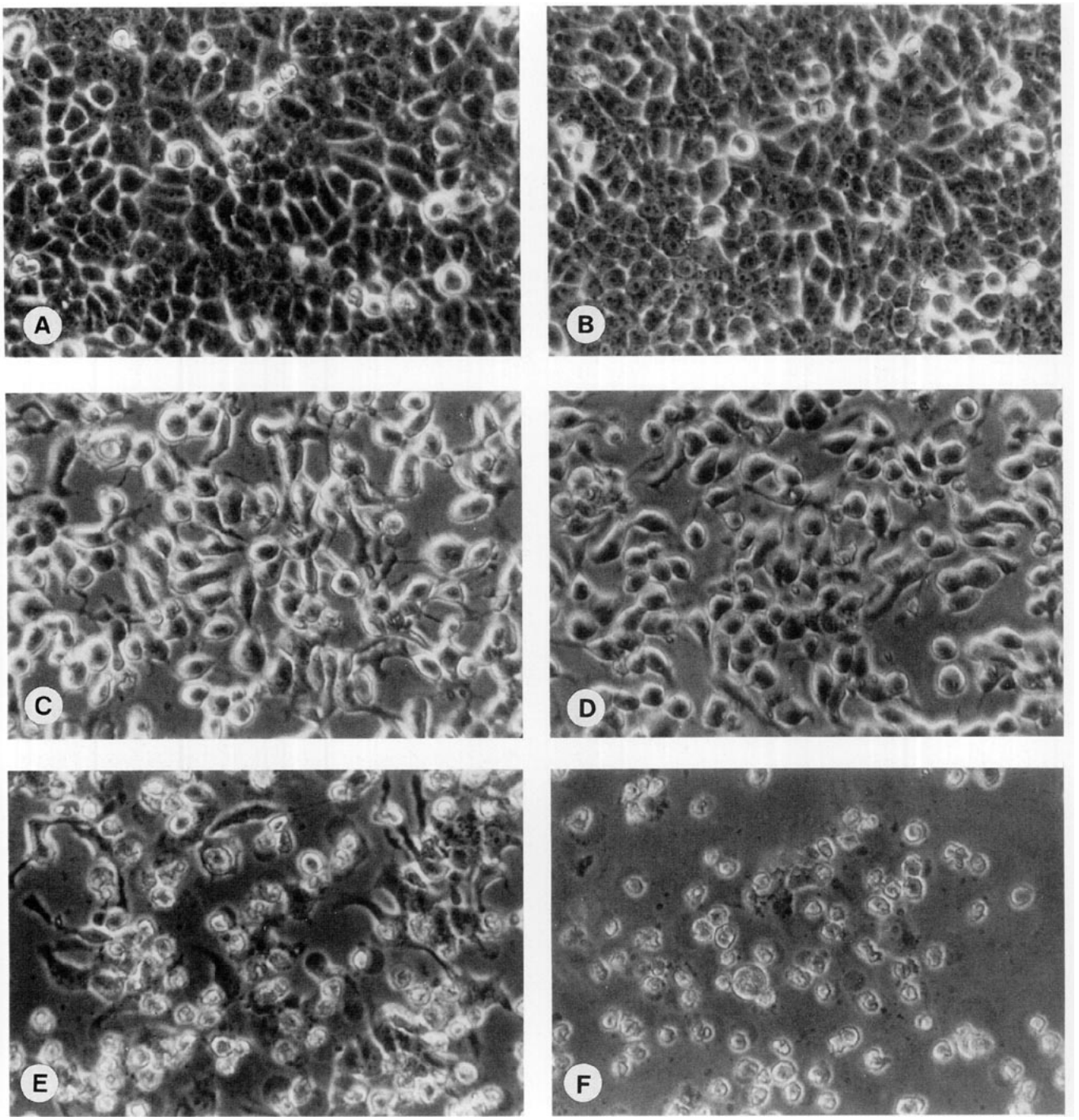

FIG. 1. Expression of a functional p68 kinase by WR68K induces severe cell shrinkage and cell death. Monolayers of HeLa cells grown in 24-well limbro plates with Dulbecco's Modified Eagle's Medium containing 10% newborn calf serum were mock-infected, referred as uninfected cells, (A, B) or infected with either $WR68K_{K\rightarrow R}$ (C, D) or WR68K (E, F) at 5 plaque-forming-units per cell, in the absence (A, C, E) or presence (B, D, F) of 1.5 m*M* IPTG. At 48 hr pi, cultures were examined by phase-contrast microscopy.

readily observed when expression of p68 kinase was induced by IPTG (Fig. 2F). At 48 hr pi, most of the cells infected with WR68K in the presence of IPTG contained apoptotic nuclei, and even in the absence of IPTG condensed nuclei were observed in small-rounded, apoptotic cells (data not shown).

One of the distinct characteristics that distinguishes apoptosis from any other type of cell death is the cleavage of DNA into 180-bp multimers corresponding to a nucleosomal "DNA ladder" (*4*). To document DNA fragmentation into oligonucleosomes, low molecular weight (MW) DNA was isolated and analyzed by agarose gel electrophoresis. At 24 hr pi, a DNA ladder indicative of apoptosis was observed only when the

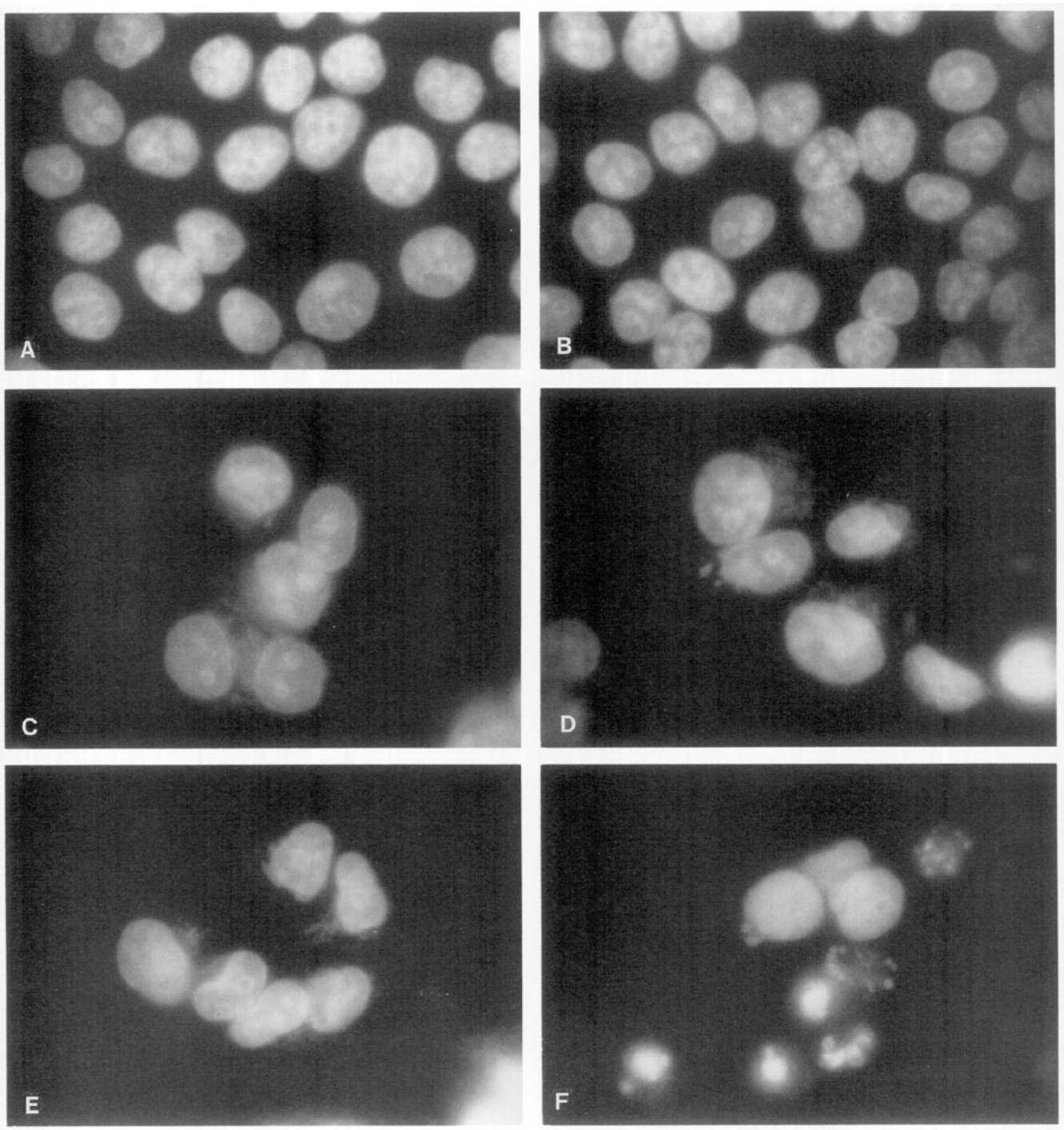

FIG. 2. Chromatin condensation and segmentation induced by p68 kinase. Subconfluent monolayers of HeLa cells grown on glass coverslips were infected as described in Fig. 1, and DNA was stained with Hoechst as previously described (*26*). Briefly, at 24 hr pi, cells were washed with phosphate-buffered saline (PBS), fixed, and permeabilized with methanol:acetone (3:1, v/v) for 10 min, washed with PBS, nuclei were stained with Hoechst 33258 (0.5 μg/ml in PBS) for 10 min in the dark, washed with PBS, mounted on slides in glycerol–citric acid phosphate buffer, pH 4.1 (9:1, v/v), and visualized under uv (oil-immersion, 100X) with fluorescent microscope (Nikon). Nuclei of mock-infected (A, B), WR68K$_{K\rightarrow R}$-infected (C, D), or WR68K-infected (E, F) cells are shown. −IPTG (A, C, E); + IPTG (B, D, F).

expression of p68 kinase was induced by IPTG in WR68K-infected cells (Fig. 3A, lane 6), but not in WR68K$_{K\rightarrow R}$-infected cells regardless of IPTG treatment (Fig. 3A, lanes 1–4). Since the endonuclease responsible for the degradation of DNA into oligonucleosomes is inhibited by Zn^{2+} (*6, 7*), we tested to see if addition of zinc ions can block DNA fragmentation. Addition of $ZnSO_4$ to the final concentration of 100 μM almost completely abolished the formation of DNA ladder (Fig. 3A, lane 8). Zinc ions were not lethal to HeLa cells at the

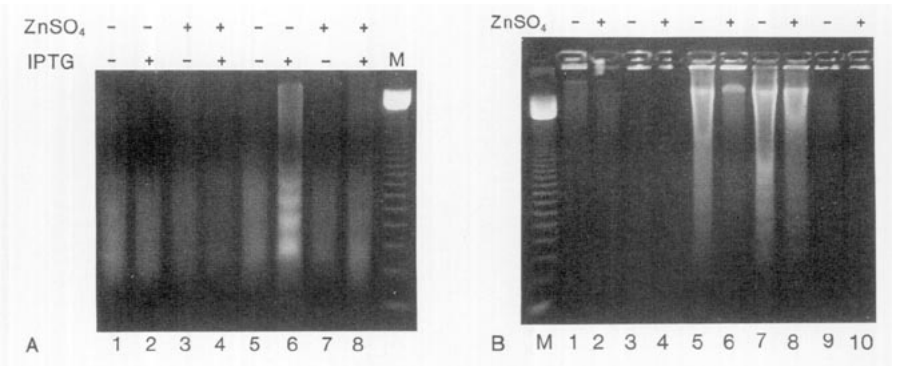

FIG. 3. DNA fragmentation induced by p68 kinase is prevented by $ZnSO_4$. Monolayers of HeLa cells grown in 100 mm plates (1×10^7 cells/plate) were infected at 5 PFU/cell with WR (wild-type VV), WR68K, WR68K$_{K\rightarrow R}$, or VV deletion mutants either lacking E3L or K3L gene products and to the duplicate plates, $ZnSO_4$ was added to the media to the final concentration of 100 μM. Cells were scraped and collected in media at either 24 or 48 hr pi, washed once with PBS, and resuspended in 1 ml of lysis solution (20 m*M* Tris–HCl, pH 8, 10 m*M* EDTA, pH 8, and 0.5% Triton X-100). After incubation on ice for 10 min, the lysates were subjected to centrifugation at 12,000 rpm for 10 min and unsedimented, low molecular weight DNA in the supernatant was extracted with an equal volume of phenol/chloroform for 1 hr at 4°. After fractionation by centrifugation, ammonium acetate was added to the aqueous phase to the final concentration of 2 *M* and DNA was precipitated with 2 vol of ethanol at −20° overnight. DNA was resuspended in 18 μl of H_2O, treated with RNase A (1 mg/ml) at 37° for 1 hr and total DNA was analyzed on 1.5% agarose gel containing 0.1 μg/ml of ethidium bromide. (A) Low molecular weight DNA isolated at 24 hr pi from cells infected with WR68K$_{K\rightarrow R}$ (lanes 1–4) or WR68K (lanes 5–8); −IPTG (lanes 1, 3, 5, 7), + IPTG (lanes 2, 4, 6, 8). (B) Low molecular weight DNA isolated at 24 hr pi from mock-infected (uninfected) (lanes 1–2), or from cells infected with WR (lanes 3–4), or E3L deletion VV (lanes 5–6); low molecular weight DNA isolated at 48 hr pi from cells infected with E3L deletion VV (lanes 7–8) or K3L deletion VV (lanes 9–10). M, 123-bp DNA ladder (Gibco, BRL). The total low molecular weight DNA obtained from 1×10^7 cells was loaded on the gels.

concentration used, because the viability of the cells remained the same after incubation with or without 100 μM $ZnSO_4$ for 24 hr.

Since VV encodes two inhibitors of p68 kinase, E3L gene, which encodes a dsRNA binding protein (*8, 9*) and K3L gene, a homolog of eIF2α (*10, 11*), we reasoned that infection with VV mutant viruses lacking these inhibitors may activate the endogenous p68 kinase to induce apoptosis. Thus, HeLa cells were infected with the VV deletion mutants lacking E3L and K3L, and low MW DNA was isolated and analyzed by agarose gel electrophoresis. As shown in Fig. 3B, at 24 hr pi, there was DNA fragmentation in cells infected with the deletion mutant virus lacking E3L gene (Fig. 3B, lane 5), while in uninfected cells or in cells infected with wild-type VV (WR), no such DNA fragmentation was observed (Fig. 3B, lanes 1–4). More severe DNA cleavage was observed at 48 hr pi (Fig. 3B, lane 7). In contrast, cells infected with K3L deletion mutant virus did not display DNA cleavage even after 48 hr pi (Fig. 3B, lanes 9 and 10), suggesting that E3L gene product is a more potent inhibitor of p68 kinase than K3L gene product. As expected, addition of $ZnSO_4$ to the media drastically reduced DNA fragmentation in cells infected with E3L mutant virus (Fig. 3B lanes 6 and 8). As determined by phase-contrast microscopy, most of the cells infected with E3L mutant virus were detached and showed characteristic apoptotic bodies (data not shown).

In order to quantitate the amount of fragmented DNA versus nonfragmented DNA, host DNA was labeled with [^{3}H]thymidine and total DNA was analyzed by velocity sedimentation on a high salt neutral linear sucrose gradient (Fig. 4). At 48 hr pi, most high molecular weight DNA (83%) was converted to small fragments when p68 kinase was expressed in WR68K-infected cells (Fig. 4). In the absence of IPTG, a significant amount of cleaved DNA (52%) was also observed in WR68K-infected cells (Fig. 4), consistent with appearance of apoptotic cells observed at 48 hr pi in the absence of the inducer (Fig. 1E). Low molecular weight DNA was not present, irrespective of IPTG treatment, in WR68K$_{K\rightarrow R}$-infected cells at 24 hr pi (data not shown) nor at 48 hr pi (Fig. 4). On the other hand, cells infected with WR68K in the presence of IPTG displayed a significant fragmentation of DNA (66%) into low molecular weight at 24 hr pi; DNA fragmentation was not evident without the inducer IPTG at 24 hr pi (data not shown, see also Fig. 3A).

Recent studies have shown that cells expressing the mutant form of p68 kinase with inactivated kinase function, when injected into nude mice, develop a malignant tumor (*12, 13*). Hence, it has been proposed that p68 kinase could serve as a tumor suppressor gene (*12, 13*), although the underlying mechanism has not been defined. In addition, p68 kinase has been implicated in the antiproliferative effects of IFNs (*14*) and indeed, expression of human p68 kinase in yeast causes growth arrest phenotype (*15*). The results reported in this investigation demonstrate that expression and activation of p68 kinase, which results in inhibition of protein synthesis, lead to apoptosis. The cell death phenomenon is physiological and is not due to overexpression of p68 kinase, since cells infected with

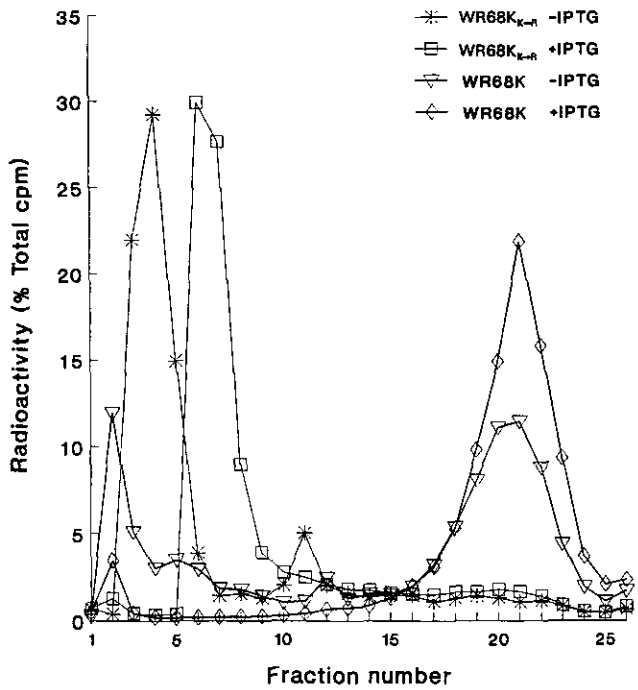

FIG. 4. Quantitation of DNA fragmentation by high salt linear sucrose gradient analysis. Subconfluent monolayers of HeLa cells grown in 12-well limbro plates were labeled with 1 μCi/ml of [^{3}H]thymidine (ICN) for 20 hr. Labeled media were replaced with unlabeled media and cells were infected (5 PFU/cell) with either WR68K$_{K\rightarrow R}$ (thin line) or WR68K (thick line). At 48 hr pi, cells were collected in media by trypsinization, washed in PBS, resuspended in 0.5 ml of PBS, and 2×10^5 cells were loaded on top of a sucrose gradient. Sedimentation analysis of DNA from cell lysates on a 15–30% (w/v) linear, neutral sucrose gradient in high salt was done as previously described (*27*). Counts (cpm) in each fractions are expressed as the percentage of total radioactivity of the corresponding sample. The top of the gradient is the highest numbered fraction and the lowest numbered fraction is the bottom of the gradient.

E3L mutant virus also developed apoptosis with DNA fragmentation (Fig. 3B). Moreover, apoptosis mediated by p68 kinase is not restricted to HeLa cells as it was also observed in cells of other origins, monkey (BSC-40) and mouse (LTK$^-$) (data not shown). In view of our findings, it is possible that some of the cytotoxic effects of IFN on many tumor and normal cells (*16–18*) are due to activation of p68 kinase. Thus, we propose that p68 kinase mediates, at least in part, antitumor effects of IFNs by promoting cell death and this effect could also explain the role of p68 kinase as a tumor suppressor gene. Because the IFN-mediated effect can vary with the cell origin this may explain our inability to detect DNA fragmentation in uninfected HeLa cells treated for 48 hr with up to 10^3 U/ml of human IFN-α, while we have observed some ($\leq$30%) DNA fragmentation in uninfected mouse L-929 cells treated with 10^3 U/ml of murine IFN-α/β (unpublished observations). Interestingly, another tumor suppressor gene, p53, has also been shown to induce apoptosis in mouse myeloid leukemia cell line (*19*) and in human colon tumor cell line (*20*). To our knowledge, p68 kinase is a second example of a tumor suppressor gene to cause apoptosis other than p53, and this strongly supports the notion that other tumor suppressor genes may also function to promote cell death (*19*).

At present, how p68 kinase mediates apoptosis is unclear. One possibility is that inhibition of protein synthesis mediated by p68 kinase may be responsible. In support of this hypothesis, there are reports that cycloheximide, an inhibitor of protein synthesis, also triggers apoptosis in certain cell lines (*21, 22*). Moreover, a proto-oncogene, c-myc, when expressed constitutively, triggers apoptosis when cells are deprived of serum or in the presence of growth-inhibiting drugs, such as cycloheximide (*23*). Thus, p68 kinase could potentially serve as a growth-inhibiting agent, and in conjunction with other gene(s) such as c-myc, might trigger apoptosis. Alternatively, p68 kinase could play a direct role in the apoptotic pathway through phosphorylation of an unknown substrate(s). The fact that there are at least two cellular inhibitors of p68 kinase (*24, 25*) indicates that the activity of the kinase must be tightly regulated. It also implies that there are natural, endogenous activators of p68 kinase and suggests that there may be more substrates of the kinase other than eIF2α. Thus, any events that lead to derepression of p68 kinase activity, such as inactivation of the endogenous inhibitor(s) of the kinase, could perhaps trigger cell death. In this regard, identification of other natural substrates of p68 kinase will shed some light into the mechanism of apoptosis.

ACKNOWLEDGMENTS

We thank Dr. E. Paoletti for kindly providing E3L and K3L deletion mutant vaccinia virus, Dr. G. K. Ojakian for help with phase-contrast microscopy and Drs. J. A. Lewis, R. Bablanian, J. R. Rodriquez, D. Rodriguez, and M. Diaz-Guerra for helpful discussions and critical review of the manuscript. This investigation was supported, in part, by NIH. This work was presented at the IX International Congress of Virology, Glasgow, August 1993.

REFERENCES

1. PESTKA, S., LANGER, J. A., ZOON, K. C., and SAMUEL, C. E., *Annu. Rev. Biochem.* **56**, 727–777 (1987).
2. HOVANESSIAN, A. G., *J. Interferon Res.* **11**, 199–205 (1991).
3. LEE, S. B., and ESTÉBAN, M., *Virology* **193**, 1037–1041 (1993).
4. WYLLIE, A. H., KERR, J. F. R., and CURRIE, A. R., *Int. Rev. Cytol.* **68**, 251–306 (1980).
5. WILLIAMS, G. T., *Cell* **65**, 1097–1098 (1991).
6. DUKE, R. C., CHERVENAK, R., and COHEN, J. J., *Proc. Natl. Acad. Sci. USA* **80**, 6361–6365 (1983).
7. MEYAARD, L., OTTO, S. A., JONKER, R. R., MIJNSTER, M. J., KEET, R. P. M., and MIEDEMA, F., *Science* **257**, 217–219 (1992).
8. CHANG, H-W., WATSON, J. C., and JACOBS, B. L., *Proc. Natl. Acad. Sci. USA* **89**, 4825–4829 (1992).
9. DAVIES, M. V., CHANG, H-W., JACOBS, B. L., and KAUFMAN, R. J., *J. Virol.* **67**, 1688–1692 (1993).

10. BEATTIE, E., TARTAGLIA, J., and PAOLETTI, E., *Virology* **183,** 419–422 (1991).
11. CARROLL, K., ELROY-STEIN, O., MOSS, B., and JAGUS, R., *J. Biol. Chem.* **268,** 12837–12842 (1993).
12. KOROMILAS, A. E., ROY, S., BARBER, G. N., KATZE, M. G., and SONENBERG, N., *Science* **257,** 1685–1689 (1992).
13. MEURS, E. F., WATANABE, Y., KADEREIT, S., BARBER, G. N., KATZE, M. G., CHONG, K., WILLIAMS, B. R. G., and HOVANESSIAN, A. G., *Proc. Natl. Acad. Sci USA* **90,** 232–236 (1993).
14. WOOD, J. N., and HOVANESSIAN, A. G., *Nature* **282,** 74–76 (1979).
15. CHONG, K. L., FENG, L., SCHAPPERT, K., MEURS, E. F., DONAHUE, T. F., FRIESEN, J. D., HOVANESSIAN, A. G., and WILLIAMS, B. R. G., *EMBO J.* **11,** 1553–1562 (1992).
16. ITO, M., and BUFFETT, R. F., *J. Natl. Cancer Inst.* **66,** 819–825 (1981).
17. TYRING, S. K., KLIMPEL, G. R., FLEISCHMANN, W. R., JR., and BARON, S., *Int. J. Cancer* **30,** 59–64 (1982).
18. TYRING, S. K., KLIMPEL, G. R., BRYSK, M., GUPTA, V., STANTON, G. J., FLEISCHMANN, W. R., JR., and BARON, S., *J. Natl. Cancer Inst.* **73,** 1067–1073 (1984).
19. YONISH-ROUACH, E., RESNITZKY, D., LOTEM, J., SACHS, L., KIMCHI, A., and OREN, M., *Nature* **352,** 345–347 (1991).
20. SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SORDAT, B., and COSTA, J., *Proc. Natl. Acad. Sci. USA* **89,** 4495–4499 (1992).
21. WARING, P. J., *J. Biol. Chem.* **265,** 14476–14480 (1990).
22. MARTIN, S. J., LENNON, S. V., BONHAM, A. M., and COTTER, T. G., *J. Immunol.* **145,** 1859–1867 (1990).
23. EVAN, G. I., WYLLIE, A. H., GILBERT, C. S., LITTLEWOOD, T. D., LAND, H., BROOKS, M., WATERS, C. M., PENN, L. Z., and HANCOCK, D. C., *Cell* **69,** 119–128 (1992).
24. JUDWARE, R., and PETRYSHYN, R., *Mol. Cell. Biol.* **11,** 3259–3267 (1991).
25. LEE, T. G., TOMITA, J., HOVANESSIAN, A. G., and KATZE, M. G., *J. Biol. Chem.* **267,** 14238–14243 (1992).
26. ESTEBAN, M., *J. Virol.* **21,** 796–801 (1977).
27. ESTEBAN, M., and HOLOWCZAK, J. A., *Virology* **78,** 57–75 (1977).