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Identification of a Rel-Related Protein in the Nucleus during the S Phase of the Cell Cycle

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The c-*rel* proto-oncogene encodes a 75-kDa protein ($p75^{c\text{-}rel}$) which is present in the cytosol of chick embryo fibroblasts (CEF) associated with a distinct set of cellular proteins with molecular masses of 40, 115, and 124 kDa. CEF cultures arrested in S phase of the cell cycle, or enriched for G_2 or mitotic cells, were examined to determine whether the expression of c-*rel* was altered during the cell cycle. Levels of $p75^{c\text{-}rel}$ remained constant in all portions of the cell cycle examined; however, a Rel-related protein with an apparent molecular mass of 64 kDa was detected in nuclei of S-phase cells. As cells enter G_2, the level of this protein in the nucleus decreases. This protein reacts with antiserum generated against the carboxy terminus of $p75^{c\text{-}rel}$ in radioimmunoprecipitations and Western immunoblot experiments and was also detected in a Western immunoblot with antiserum generated against the first 161 amino acids of $pp59^{v\text{-}rel}$. Thus, unlike other Rel/NF-κB family members, p64 has carboxy-terminal homology with c-Rel. The majority of peptides generated by partial proteolytic cleavage of p64 are shared with peptides generated by digestion of $p75^{c\text{-}rel}$ and/or $pp59^{v\text{-}rel}$. We suggest that this protein represents a new member of the Rel family of transcription factors and is located in the nucleus of avian fibroblasts during S phase of the cell cycle.

The proto-oncogene c-*rel* encodes a 75-kDa protein ($p75^{c\text{-}rel}$) which is a member of the Rel/NF-κB family of transcription factors (38, 53). This family includes the product of the v-*rel* oncogene carried by reticuloendotheliosis virus (REV-T), the *Drosophila melanogaster* maternal morphogen Dorsal, the various subunits of the mammalian transcription complex NF-κB, human p49, and murine RelB and its human homolog I-Rel (24, 34, 42, 46–48, 55). These proteins share homology within approximately 350 amino acids near the amino terminus (the Rel domain), and all except v-Rel and c-Rel are completely divergent at their carboxy termini (10, 34, 42, 47, 48). With one exception, these proteins are able to bind DNA in vitro to the consensus NF-κB binding site (κB site) as homo- or heterodimers (4, 6, 34, 42, 44, 47, 48). Dorsal has not been shown to form heterodimers, nor has it been shown to bind the consensus κB site; however, it does bind a related site in the *zen* promoter (31). Recent data utilizing degenerate oligonucleotides indicate that different combinations of Rel family members preferentially bind divergent κB-related motifs (44). Which Rel family members heterodimerize in vivo and are physiologically active is still a matter of speculation.

NF-κB was initially identified in lymphoid cells as a heterodimer composed of a 50-kDa subunit (p50) which is derived from a 110-kDa precursor by proteolytic cleavage and a 65-kDa subunit (p65) (2–4, 23, 25, 34, 58). This DNA-binding complex is sequestered in the cytosol by association with the inhibitory protein IκB (2, 3). $p75^{c\text{-}rel}$ is found in the cytosol of avian lymphocytes associated with three major proteins (40, 115, and 124 kDa) (15, 19, 35, 40). The 40-kDa protein is functionally related to the β-form of IκB, an inhibitor of c-Rel/NF-κB (21, 33). The 124-kDa Rel-associated protein is the avian homolog of the murine 110-kDa precursor of p50 (29). When lymphocytes are activated, it is proposed that IκB dissociates from NF-κB, resulting in translocation of NF-κB to the nucleus (3). Binding sites for NF-κB reside in the regulatory regions of many viral enhancers, including the human immunodeficiency virus long terminal repeat and a variety of diverse cellular genes including those encoding the β-chain of the T-cell receptor and the proto-oncogenes c-*myb* and c-*myc* (22, 32). It is unknown which of these sites are also bound by c-Rel; however, c-Rel has been shown to bind a κB-related site in an intronic enhancer of the human gamma interferon gene (52).

All of the Rel/NF-κB family members except $p75^{c\text{-}rel}$ have been observed in the nucleus of normal cells under the appropriate conditions. $p75^{c\text{-}rel}$ has only been observed to translocate to the nucleus after Zn^{2+} stimulation of REV-T-transformed avian lymphoid cells (56). $p75^{c\text{-}rel}$ does not translocate to the nucleus of cells even when overexpressed. Deletion of 103 amino acids from the carboxy terminus of the c-Rel protein (giving a protein of 63 kDa) allows nuclear translocation of the protein in cells overexpressing this truncated form (15). Since $p75^{c\text{-}rel}$ has been shown to be a transcription factor, it would be expected that the protein would be found in the nucleus of normal cells under the appropriate conditions (21, 30).

NF-κB DNA-binding activity is rapidly induced after stimulation of quiescent 3T3 cells with serum or growth factors (5, 43). Near the end of G_1, this activity decreases substantially, and it cannot be detected in proliferating cells. A different NF-κB-like activity is induced under the same conditions but persists in proliferating cells. c-*rel* is induced by serum in quiescent fibroblasts, and the level of c-*rel* transcripts decreases to nearly basal levels 3 h after stimulation (12). This suggests that c-*rel* could be involved in the G_0-to-G_1 transition. These observations suggest a role for Rel family members during cellular proliferation. In this paper we describe the existence of a new 64-kDa Rel family member which is located in the nucleus of avian fibroblasts

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in S phase. Unlike previously described Rel family members, p64 shares extensive carboxy-terminal homology with $p75^{c\text{-}rel}$.

MATERIALS AND METHODS

Growth and synchronization of cells. Primary chicken embryo fibroblast (CEF) cultures prepared from 11-day-old chicken embryos (SPAFAS, Norwich, Conn.) were grown in Eagle's minimal essential medium supplemented with 6% newborn calf serum and 2% serum plus (Hazelton, Lenexa, Kans.), penicillin (200 U/ml), and streptomycin (100 μg/ml). Subconfluent cultures were treated with the nucleotide reductase inhibitor hydroxyurea (1 mM), the DNA polymerase α inhibitor aphidicolin (5 μg/ml), or the microtubule poison methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate (nocodazole; 0.4 μg/ml) (all available from Sigma, St. Louis, Mo.) for 24 to 27.5 h (16, 57). In one experiment, cells treated with hydroxyurea were washed three times with phosphate-buffered saline (PBS) to release the cells from drug arrest and then overlaid again with fresh media.

The REV-T-transformed avian lymphoid cell line RECC-UT C41 was maintained in RPMI 1640 medium supplemented with 6% newborn calf serum, 2% serum plus, and antibiotics. All cells were grown in a 37°C humidified incubator with 5% CO_2.

Flow cytometry. For each experiment, 10^6 cells were fixed in 700 μl of 35% ethanol in PBS. Immediately prior to analysis by flow cytometry, 250 μl of the fixed sample was removed, filtered through glass wool, treated with RNase A, and stained with propidium iodide (16). Cytometric analysis was performed on a Coulter EPICS V fluorescence-activated cell sorter.

Subcellular fractionation. Nuclear preparations were prepared as described by Hastie and Mahy (27). All solutions contained the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and aprotinin (100 KIU/ml). Briefly, CEF were swollen in hypotonic buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 1.6 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM triethanolamine) and ruptured by Dounce homogenization. Cell lysates were centrifuged at 10,000 × *g* to provide a crude nuclear pellet and a cytosolic fraction. The nuclear pellet was suspended in 11% sucrose containing 1 mM $MgCl_2$ and underlaid with 58.5% sucrose containing 1 mM $MgCl_2$. Nuclei were collected by centrifugation at 75,000 × *g* for 40 min and resuspended in 11% sucrose. Nonidet P-40 was added to a final concentration of 1%. Nuclei were incubated in this solution for 1 min and washed twice in 11% sucrose. High-detergent (1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate) immunoprecipitation assay buffer (20 mM Tris [pH 7.5], 2 mM EDTA, 150 mM NaCl) containing the protease inhibitors phenylmethylsulfonyl flouride (1 mM) and aprotinin (100 KIU/ml) was added, and cells were either homogenized or subjected to repeated freeze-thaw cycles. The detergent was then diluted with either 150 μl of low-detergent (0.5% Triton X-100) IP buffer or hypotonic buffer. Membranes were removed by centrifugation, and protein concentrations were determined by the method of Bradford with the Bio-Rad protein microassay (Richmond, Calif.) (11). The nucleoplasm preparation did not contain detectable levels of the cytosolic enzyme lactate dehydrogenase (7).

Immunologic reagents. Antiserum against $p75^{c\text{-}rel}$ was prepared by fusion of a 696-bp region of c-*rel* in frame with the pATH10 expression vector. Expression of this vector generates a protein with a portion of the *Escherichia coli trpE* gene fused to amino acids 347 to 579 of $p75^{c\text{-}rel}$. This portion of c-Rel is shown in Fig. 1A (15). Antiserum against $pp59^{v\text{-}rel}$ was prepared by fusion of the portion of the gene encoding amino acids 1 to 161 into an expression vector. Proteins expressed in *E. coli* from both of these vectors were used to immunize rabbits (38, 60).

Radiolabeling and immunoprecipitation. Cultures incubated in the presence of hydroxyurea or nocodazole for 20.5 h were washed twice with PBS and starved in methionine- and cysteine-free medium containing the appropriate drugs and 5% fetal calf serum for 3.5 h (16, 57). [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml; Express label, NEN DuPont, Boston, Mass.) were added to the cultures, which were then incubated for 3.5 h. Cultures were then washed twice in PBS and prepared for subcellular fractionation or lysed in low-detergent IP buffer containing protease inhibitors.

Radioimmunoprecipitations were performed on whole-cell lysates, cytosolic fractions, or nucleoplasm by using antiserum generated against amino acids 347 to 579 of $p75^{c\text{-}rel}$ after lysates were precleared with normal rabbit serum. The final immunoprecipitate was formed by addition of 25 μl of a 50% slurry of protein A-Sepharose (Pharmacia, Piscataway, N.J.) prepared in low-detergent immunoprecipitation buffer.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (37). Radiolabeled proteins were visualized by exposure to XAR-5 X-ray film (Eastman Kodak, Rochester, N.Y.). Molecular weight estimates were based on the relative migration of marker proteins (Sigma, St. Louis, Mo.; Pharmacia, Piscataway, N.J.).

Western immunoblots. Proteins resolved by SDS-PAGE were electrophoretically transferred to 0.1-μm-pore-size nitrocellulose (Schleicher and Schuell, Keene, N.H.) in Tris-glycine buffer (25 mM Tris-HCl [pH 8.3], 150 mM glycine, 15% methanol) and then stained with Ponceau S (0.5% in 1% acetic acid) to confirm that equivalent amounts of protein had transferred to the nitrocellulose (13, 49). The filters were treated with blocking buffer consisting of either 10% nonfat dry milk or 5% bovine serum albumin in Tris-saline buffer (TSB; 0.9% NaCl, 10 mM Tris-HCl [pH 7.4]). Filters were incubated with anti-c-Rel antiserum (various dilutions in blocking buffer) at 4°C overnight or with anti-v-Rel antiserum (1:250 dilution in blocking buffer) for several hours at room temperature. Primary antibody was detected with goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate in blocking buffer. The filters were developed in 150 μg of 5-bromo-4-chloro-3-indolyl-phosphate per ml and 300 μg of nitroblue tetrazolium per ml in 50 mM $NaHCO_3$ (pH 9.8) with 5 mM $MgCl_2$ and 150 mM NaCl.

In vitro transcription and translation. The c-*rel* gene (gift of D. Simmons) or c-*rel* in which the 40 carboxy-terminal amino acids were deleted was cloned into a pBluescript vector (Stratagene, La Jolla, Calif.). This allowed simultaneous in vitro transcription and translation of these genes by following the instructions enclosed with the kit (TnT-coupled reticulocyte lysate system; Promega, Madison, Wis.).

Proteolytic mapping. Proteins were mapped by partial proteolysis as described by Cleveland et al. except that protein bands were excised from unfixed SDS-polyacrylamide gels and digested with 0.5 μg of staphylococcal V8 protease (Sigma) (18). Signal intensity was enhanced by soaking the gel in a PPO solution (525 ml of dimethylsulfoxide, 100 g of 2,5-diphenyloxazole) prior to drying.

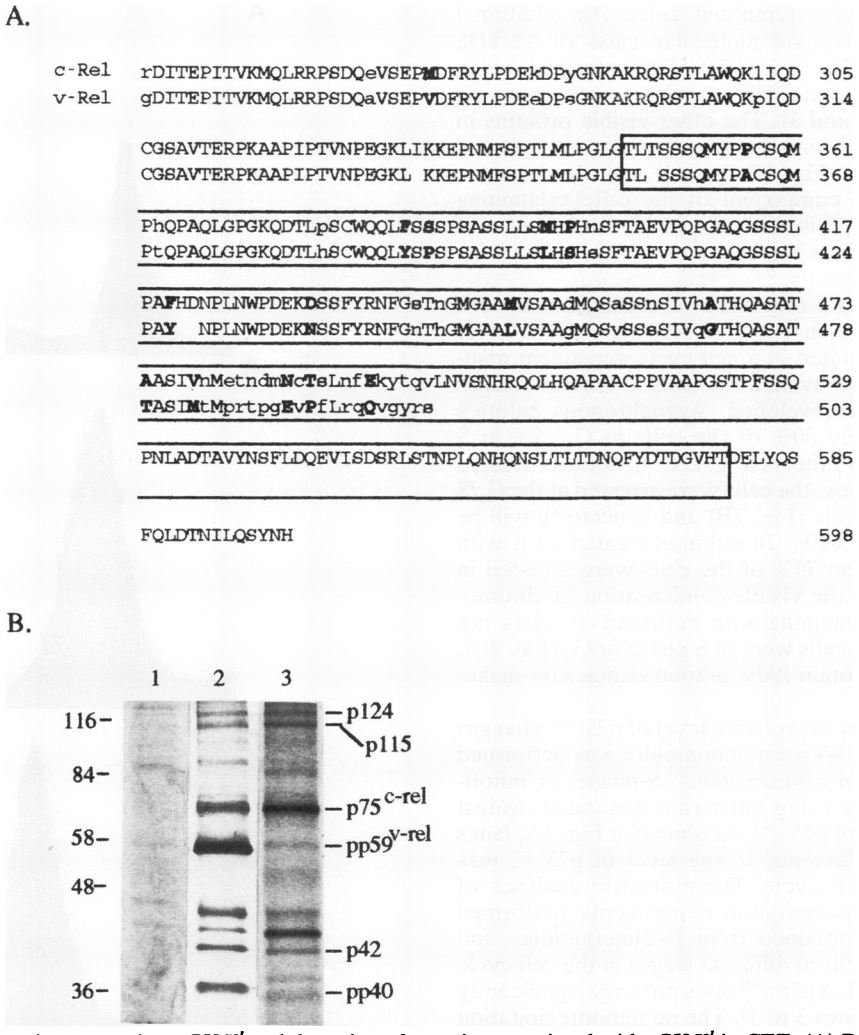

FIG. 1. Production of antiserum against p75$^{c\text{-}rel}$ and detection of proteins associated with p75$^{c\text{-}rel}$ in CEF. (A) Boxed area depicts amino acids of p75$^{c\text{-}rel}$ to which antiserum was produced. The upper amino acid sequence is that of p75$^{c\text{-}rel}$ from residue 300 to residue 598 (the end of the protein) (14). The lower amino acid sequence is that of pp59$^{v\text{-}rel}$ from residue 312 to residue 503 (54). Regular type represents identical amino acids, and boldface type represents conservative amino acid changes between v-Rel and c-Rel. Lowercase letters represent nonconservative amino acid substitutions between v-Rel and c-Rel, and spaces in the lower sequence represent deletions in v-Rel which occurred during transduction. (B) Proteins associated with p75$^{c\text{-}rel}$ in CEF. CEF or transformed avian lymphoid cells were starved for methionine and cysteine and then labeled with 70 μg of [^{35}S]methionine per ml and 30 μCi of [^{35}S]cysteine per ml for 3 h. Whole-cell lysates were prepared in radioimmunoprecipitation buffer containing 0.5% Triton X-100. Proteins were precipitated with normal rabbit serum or antiserum against p75$^{c\text{-}rel}$. Proteins were separated by SDS-PAGE (9% acrylamide), and the gels were autoradiographed. Lanes 1 and 2, lysates from REV-T-transformed avian lymphoid cells precipitated with normal rabbit serum or antiserum against p75$^{c\text{-}rel}$; lane 3, lysate from CEF precipitated with antiserum recognizing p75$^{c\text{-}rel}$. Molecular masses are given in kilodaltons.

RESULTS

p75$^{c\text{-}rel}$ is associated with a unique set of proteins in CEF. p75$^{c\text{-}rel}$ in normal avian bursal lymphocytes is found in large protein complexes associated with a distinct set of cellular proteins. Those present in greatest abundance are pp40, p115, and p124, although other proteins were also detected (15, 19, 35, 40). To determine whether p75$^{c\text{-}rel}$ is associated with these same proteins in nonlymphoid cells, REV-T-transformed avian lymphoid cells and asynchronously growing CEF were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Proteins from extracts of whole cells prepared in low-detergent immunoprecipitation buffer were immunoprecipitated with antiserum generated against the carboxy terminus of p75$^{c\text{-}rel}$. Figure 1A identifies the region of avian p75$^{c\text{-}rel}$ which was employed to prepare this antiserum (amino acids 347 to 579) (15, 54). These amino acids lie just beyond the region of homology shared among Rel/NF-κB family members. This antiserum precipitated a 75-kDa protein and pp59$^{v\text{-}rel}$ in REV-T-transformed avian lymphoid cells (Fig. 1B, lane 2). The pp40, p115, and p124 proteins which are complexed with p75$^{c\text{-}rel}$ in lymphoid cells were also coimmunoprecipitated. The 75-kDa protein has previously been shown to be p75$^{c\text{-}rel}$ (38, 53). A protein which migrates at the same molecular weight was precipitated from normal CEF by this antiserum (Fig. 1B, lane 3). In addition, pp40, p115, and p124 coprecipitated with p75$^{c\text{-}rel}$ from radiolabeled extracts of normal CEF (Fig. 1B, lane 3). It is apparent that less pp40 is present in CEF than in

REV-T-transformed avian lymphoid cells. An additional protein with an approximate molecular mass of 42 kDa coprecipitated with p75$^{c\text{-}rel}$ in REV-T-transformed avian lymphoid cells and normal CEF when this antiserum was used (Fig. 1B, lanes 2 and 3). The other visible proteins in the immunoprecipitation experiments were also precipitated with preimmune serum (Fig. 1B, lane 1) and are therefore presumed not to be a component of the c-Rel-containing complexes. Thus, p75$^{c\text{-}rel}$ is associated with the same set of cellular proteins in CEF as has been previously reported for avian lymphoid cells.

p75$^{c\text{-}rel}$ is expressed at a constant level throughout the cell cycle. To establish whether the expression of the c-*rel* proto-oncogene is regulated in a cell cycle-dependent manner, procedures to arrest avian fibroblasts in different stages of the cell cycle were developed. Asynchronous cultures contained approximately 80% of the cells in G_1, 5% in S phase, and 15% in G_2 or mitosis (Fig. 2A). In cultures treated for 24 h with hydroxyurea, the cells were arrested at the G_1/S boundary of the cell cycle (Fig. 2B) and henceforth will be referred to as S-phase cells. In cultures treated 24 h with nocodazole, greater than 75% of the cells were arrested in mitosis on the basis of the visible condensation of chromosomes observed after staining with mithramycin (data not shown). The remaining cells were in S phase or G_2 (Fig. 2C). Repeated attempts to obtain 100% mitotic samples by shake-off were unsuccessful.

To determine whether the relative level of p75$^{c\text{-}rel}$ changes during the cell cycle, a Western immunoblot was performed on whole-cell lysates of asynchronous, S-phase, or mitotically enriched CEF by using antiserum generated against amino acids 347 to 579 of p75$^{c\text{-}rel}$. As shown in Fig. 3A, lanes 2 to 4, no apparent difference in the level of p75$^{c\text{-}rel}$ was detected during the cell cycle. Densitometric analyses of p75$^{c\text{-}rel}$ from immunoprecipitation experiments performed on whole-cell lysates obtained from [^{35}S]methionine- and [^{35}S]cysteine-labeled cells at different stages in the cell cycle confirmed that the level of p75$^{c\text{-}rel}$ does not vary significantly (15 to 30%) (Fig. 3B, lanes 3 to 5). The immunoprecipitation experiments also demonstrate that the composition and relative abundance of the proteins associated with p75$^{c\text{-}rel}$ do not vary during the cell cycle. The only exception is a reduction in the level of p42 in the p75$^{c\text{-}rel}$ complex in mitotic cells (Fig. 3B, lane 5).

A Rel-related protein is detected in the nucleus during S phase of the cell cycle. The product of the c-*rel* proto-oncogene has only been detected in the cytosol of normal unstimulated avian cells despite the fact that it possesses DNA-binding activity in vitro (21, 30). To determine whether p75$^{c\text{-}rel}$ is present in the nucleus at a specific point during the cell cycle, cytosolic and nuclear fractions were prepared from asynchronous CEF cultures and cultures arrested in S phase. These fractions were prepared in the presence of protease inhibitors, and the samples were immediately placed in SDS-PAGE sample buffer to reduce the possibility of proteolytic degradation. To obtain nuclei which were free of cytosolic contamination (as determined by lactate dehydrogenase assay), rigorous cellular fractionation was performed. In this process, large numbers of nuclei were lost, requiring that the experiments be standardized by use of equivalent protein. In the following experiments, the nuclear protein was derived from approximately 100 times the number of cells as was the cytosolic protein. In the nucleus of S-phase CEF, a protein with an apparent molecular mass of 64 kDa (p64) was detected by Western blot with antiserum prepared against p75$^{c\text{-}rel}$. Full-length p75$^{c\text{-}rel}$ was observed

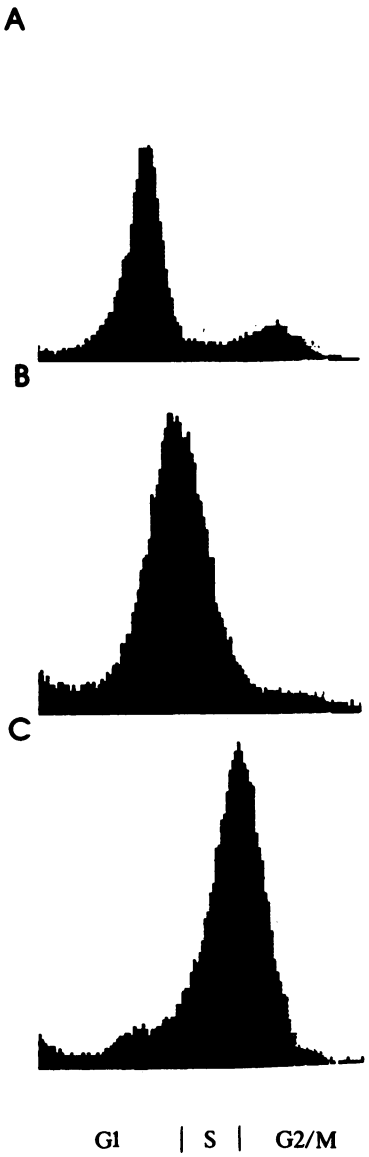

FIG. 2. CEF arrested at different stages of the cell cycle. Cells were fixed with ethanol and stained with 4.5 μg of propidium iodide. Flow cytometry was performed on a Coulter EPICS V fluorescence-activated cell sorter at a wavelength of 488 nm. Histograms of cell number (*y* axis) versus DNA content (*x* axis) were generated via Coulter MDADS software. On the basis of DNA content, the portions of the histograms representing G_1, S phase, or G_2/M are marked under panel C. G_2 and mitosis cannot be distinguished by this type of analysis. (A) Asynchronously growing CEF; (B) cells treated for 24 h with 1 mM hydroxyurea; (C) cells treated for 24 h with 0.4 μg of nocodazole per ml.

only in the cytosol (Fig. 4A, lanes 4 and 5). A protein corresponding in mass to cytosolic p75$^{c\text{-}rel}$ was never detected in nuclear extracts. When equivalent amounts of protein from cytosolic or nuclear fractions of asynchronously growing CEF (which contain approximately 80% G_1 cells) were examined, p64 was not detected by Western blot in the nuclear fraction; however, an equivalent amount of p75$^{c\text{-}rel}$ is present in the cytosolic fraction (Fig. 4A, lanes 2 and 3). p64 was precipitated from the nuclear fraction of radiolabeled S-phase CEF by using antiserum generated

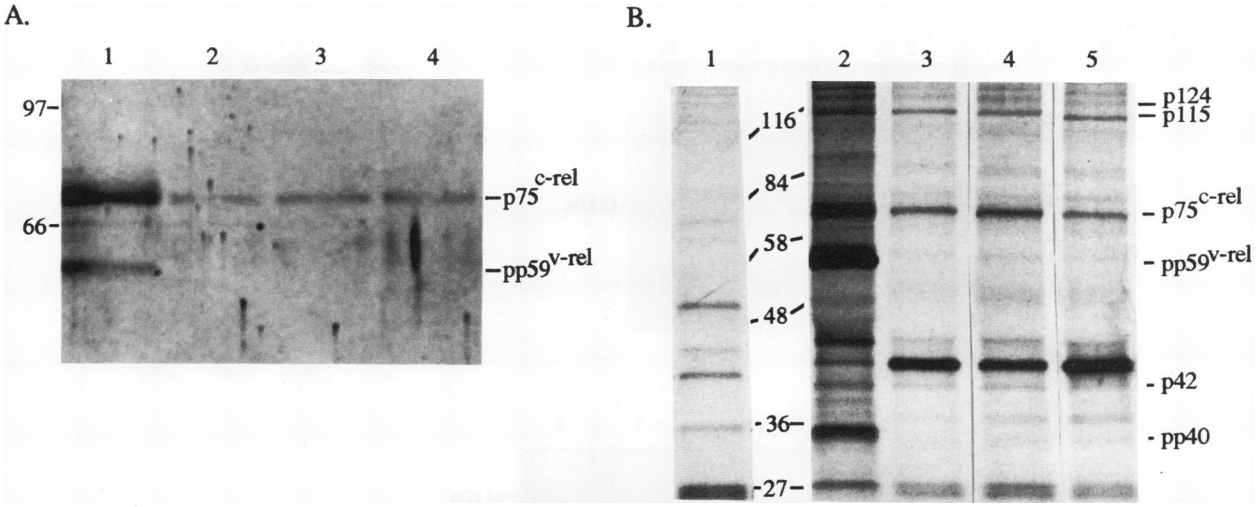

FIG. 3. $p75^{c\text{-}rel}$ is expressed at a constant level in cell cycle-arrested CEF. (A) Cells were treated as described in the legend to Fig. 2, washed once in PBS, and lysed by addition of Laemmli buffer and subsequent boiling. Total protein (15 μg) from each sample was separated by SDS-PAGE (9% acrylamide) and transferred to nitrocellulose. The immunoblot was performed as described in Materials and Methods with a 1:300 dilution of antiserum generated against amino acids 347 to 579 of $p75^{c\text{-}rel}$ as the primary antibody. Lane 1, REV-T-transformed avian lymphoid cells; lane 2, asynchronously growing CEF; lane 3, S-phase-arrested CEF; lane 4, mitotically enriched CEF. (B) Radioimmunoprecipitations were performed on 50 μg of protein from whole-cell lysates of asynchronous, S-phase, or mitotic CEF by using preimmune antiserum or antiserum generated to $p75^{c\text{-}rel}$. Proteins were resolved by SDS-PAGE (10% acrylamide). Lanes 1 and 2, proteins from REV-T-transformed avian lymphoid cells precipitated with preimmune antiserum or antiserum against $p75^{c\text{-}rel}$, respectively; lanes 3, 4, and 5, proteins from asynchronously growing, S-phase, or mitotic CEF precipitated with antiserum against $p75^{c\text{-}rel}$, respectively.

against amino acids 347 to 579 of $p75^{c\text{-}rel}$ (Fig. 4B, lane 6). Full-length $p75^{c\text{-}rel}$ was the most abundant protein immunoprecipitated from cytosolic extracts of S-phase cells by this antiserum, but some p64 was detected in cytosolic extracts (Fig. 4B, lane 4). When sufficient sample is used, p64 may also be detected by Western blot in whole-cell lysates and cytosolic fractions of S-phase CEF (data not shown).

If p64 is related to avian Rel proteins, then it should also react with antiserum generated against v-Rel. This protein, p64, has been detected in REV-T-transformed avian lymphoid cells, in which it is found in greater abundance than in CEF. Figure 7B, lanes 2 and 3, demonstrate that p64 from REV-T-transformed avian lymphoid cells and S-phase CEF are identical after partial proteolytic digestion. By using whole-cell lysates from these REV-T-transformed cells, p64 was detected in a Western immunoblot with antiserum generated against amino acids 1 to 161 of avian v-Rel (Fig. 4C, lane 2). This region of v-Rel corresponds to slightly less than one-half of the Rel homology domain. c-Rel only differs from v-Rel in this region at 4 amino acid residues, aside from the 11 amino-terminal residues of v-Rel which are derived from REV-T. The reason $pp59^{v\text{-}rel}$ reacts with this antiserum much more extensively than with either $p75^{c\text{-}rel}$ or p64 is because $pp59^{v\text{-}rel}$ is present in 10-fold excess in extracts prepared from REV-T-transformed cells. In addition, this antiserum was prepared against $pp59^{v\text{-}rel}$, which contains the highly immunogenic REV-A envelope sequences at its amino terminus. This demonstrates that p64 shares amino-terminal homology with the avian Rel proteins.

The avian homolog of IκB has been identified as the 40-kDa protein (pp40) which is associated with c-Rel in cytosolic complexes (21, 40). Although pp40 has been found associated with $pp59^{v\text{-}rel}$ in the nucleus of REV-T-transformed cells, we have not detected pp40 associated with p64 in the nucleus of CEF (20). A protein of similar apparent molecular mass as pp40 was coprecipitated from nuclei of S-phase CEF by using antiserum generated against $p75^{c\text{-}rel}$ (Fig. 4B, lane 6). This protein has a molecular mass of approximately 42 kDa and failed to precipitate from nuclear extracts with antiserum specific for avian pp40. Size exclusion chromatography indicated that the p64 protein is associated with p42 in a large-molecular-weight complex with an apparent molecular mass of 250 to 300 kDa (data not shown). The $p75^{c\text{-}rel}$ protein is not present in this complex.

To rule out the possibility that the appearance of p64 was induced by hydroxyurea, an alternate drug (aphidicolin) was used to arrest CEF in S phase. An immunoblot was performed on cytosolic and nuclear fractions from these cells and asynchronous CEF with antiserum generated against amino acids 347 to 579 of $p75^{c\text{-}rel}$. Figure 5, lanes 2 and 3, show that the level of $p75^{c\text{-}rel}$ in the cytosol was equivalent in these cells. Figure 5, lanes 4 and 5, show nuclear protein fractions from asynchronous and S-phase CEF arrested with aphidicolin. p64 was detected in the S-phase nuclei but not the nuclei from asynchronously growing CEF. Since the same observation was made with two different drugs capable of arresting CEF in the S phase of the cell cycle, it is quite unlikely that the appearance of p64 was due to drug treatment. To confirm that the appearance of p64 in the nucleus is cell cycle regulated, CEF were released from S phase by removal of the hydroxyurea and allowed to progress in the cell cycle. Approximately 30% of these cells had progressed into G_2 or mitosis 8.5 h after removal of the drug (Fig. 6A and B). A Western immunoblot performed with antiserum against $p75^{c\text{-}rel}$ demonstrated that there was a decrease in the level of p64 in the nucleus as cells progressed from S phase to G_2. Densitometric analysis of these bands showed a 22% decrease in the level of p64 in nuclei of released cells. Ponceau S staining of the nitrocellulose filter verified that equivalent amounts of protein were transferred. All of these nuclei must have been from S phase or G_2 cells, since cells

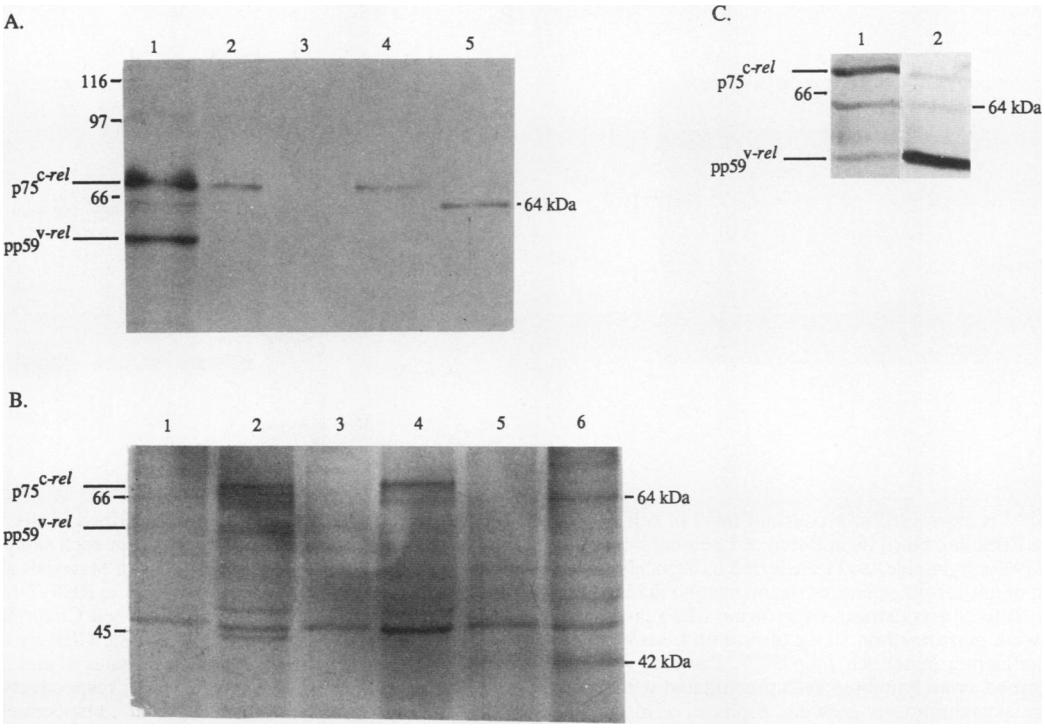

FIG. 4. A c-Rel-related protein is detected in the nucleus of S-phase-arrested CEF. Nuclei from CEF were purified by centrifugation through a 58.5% sucrose cushion followed by one wash with 11% sucrose containing 1% Nonidet P-40 and two washes with 11% sucrose. (A) Proteins (10 μg) in cytosolic and nuclear fractions from CEF cultures arrested in various stages of the cell cycle were separated by SDS-PAGE (9% acrylamide) and transferred to nitrocellulose. An immunoblot was performed as described in Materials and Methods by using antiserum generated against amino acids 347 to 579 of $p75^{c\text{-}rel}$. Lane 1, proteins in whole-cell lysate of REV-T-transformed avian lymphoid cells; lanes 2 and 3, cytosolic and nuclear fractions from asynchronously growing CEF; lanes 4 and 5, cytosolic and nuclear fractions from S-phase-arrested CEF. (B) Cytosolic and nuclear fractions from various cells were adjusted to contain 0.5% Triton X-100. Proteins (50 μg) were immunoprecipitated with normal rabbit serum or antibody generated against amino acids 347 to 579 of $p75^{c\text{-}rel}$ (3 μl) after preimmune clearing with normal rabbit serum (3 μl). Lanes 1 and 2, proteins in whole-cell lysate from REV-T-transformed avian lymphoid cells precipitated by normal rabbit serum or antibody against c-Rel; lanes 3 and 4, proteins from cytosol of S-phase-arrested CEF precipitated by normal rabbit serum or antibody to $p75^{c\text{-}rel}$; lanes 5 and 6, proteins from the nuclear fraction of S-phase-arrested CEF precipitated by normal rabbit serum or antibody against $p75^{c\text{-}rel}$. (C) Protein (50 μg) from a whole-cell lysate of REV-T-transformed avian lymphoid cells were separated by SDS-PAGE (9% acrylamide) and transferred to nitrocellulose. Immunoblots were performed as described in Materials and Methods by using various antisera. Lane 1, proteins detected with antiserum against amino acids 347 to 579 of c-Rel; lane 2, proteins detected with antiserum against amino acids 1 to 161 of v-Rel.

do not have a nuclear envelope during mitosis. (We are unable to obtain CEF arrested in G_2.)

The 64-kDa protein shares proteolytic fragments with $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$. To determine whether the 64-kDa protein recognized by the antiserum prepared against $p75^{c\text{-}rel}$ is an altered form of c-Rel or a closely related protein, partial proteolytic digestion was performed on $p75^{c\text{-}rel}$, $pp59^{v\text{-}rel}$, and p64 with staphylococcal V8 protease. The proteins used for proteolytic mapping were obtained by immunoprecipitation with antiserum against the carboxy terminus of $p75^{c\text{-}rel}$ and resolved by SDS-PAGE. Figure 7A demonstrates that $p75^{c\text{-}rel}$, p64, and $pp59^{v\text{-}rel}$ were well separated prior to excision of the protein bands for partial proteolytic digestion. This figure represents an overnight exposure of these immunoprecipitations, and although p64 from S-phase CEF is barely visible, the protein gave visible bands upon proteolytic digestion after a 6-week exposure (Fig. 7B, lane 4). The proteolytic maps were performed on p64 which was obtained from whole-cell lysates of REV-T-transformed cells or avian fibroblasts (Fig. 7B, lanes 2 and 3) or a nuclear fraction from S-phase CEF (Fig. 7B, lane 4). Comparison of these proteolytic maps demonstrates that six fragments generated from

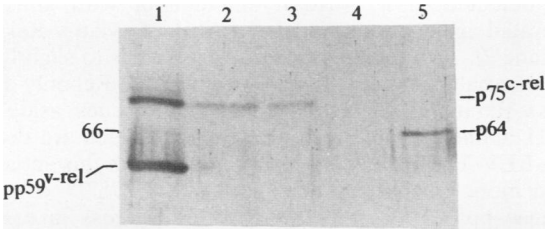

FIG. 5. The 64-kDa Rel-related protein is detected after arrest of CEF in S phase by aphidicolin. CEF were arrested in S phase by treatment with 5 μg of aphidicolin per ml. Cytosolic and nuclear fractions were prepared from asynchronous or S-phase CEF as described in Materials and Methods. Total protein (10 μg) from cytosolic or nuclear fractions or 25 μg of a whole-cell lysate from REV-T-transformed avian lymphoid cells was resolved by SDS-PAGE (7% acrylamide) and transferred to nitrocellulose. The proteins were detected with antiserum against $p75^{c\text{-}rel}$. Lane 1, whole-cell lysate from REV-T-transformed avian lymphoid cells; lanes 2 and 3, cytosolic fractions from asynchronous or aphidicolin-treated CEF; lanes 4 and 5, nuclear fractions from asynchronous or aphidicolin-treated CEF.

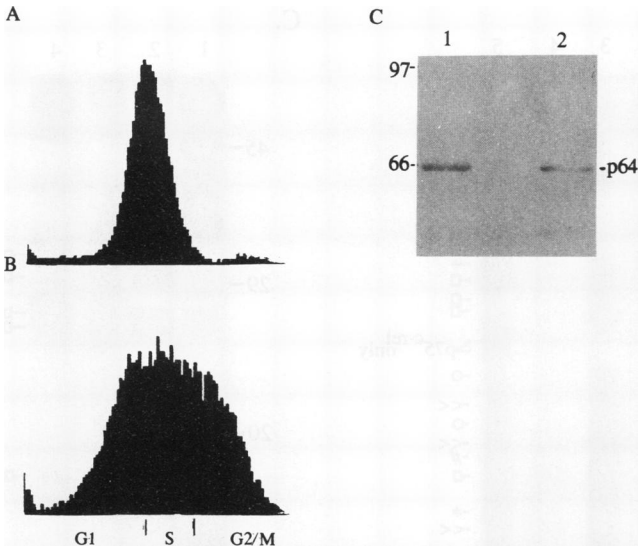

FIG. 6. The level of p64 in the nucleus decreases as cells leave S phase. CEF were treated with hydroxyurea for 24 h and then released from S-phase arrest by washing out the drug. Proteins (10 μg) from purified nuclei were separated on the same SDS-PAGE (7% acrylamide) and transferred to nitrocellulose. Proteins were detected as described previously by using antiserum against c-Rel as the primary antibody. Histograms were generated as described previously. (A) S-phase CEF generated by 24-h treatment with 1 mM hydroxyurea; (B) CEF after 8.5-h release from S-phase arrest; (C) Western immunoblots of nuclear proteins from S phase or S/G_2 CEF (8.5-h release from S-phase arrest) with antiserum against $p75^{c\text{-}rel}$.

p64 were identical to fragments generated from both $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$. Three fragments were shared by p64 and $p75^{c\text{-}rel}$ but not by $pp59^{v\text{-}rel}$. Two fragments were shared by p64 and $p59^{v\text{-}rel}$ but not by $p75^{c\text{-}rel}$. In addition, three fragments were shared by $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$ but not by p64. Two fragments were found only in $p75^{c\text{-}rel}$ and four fragments were unique to p64. Thus, 11 peptide fragments of the 15 generated from p64 by partial V8 protease digestion were identical to fragments found in either or both $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$. Since Rel family members share homology in the amino-terminal region and diverge beyond their first 350 amino acids, it is unlikely that a proteolytic map of an unrelated protein would share so many fragments with $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$. These results indicate that p64 from CEF is either an altered form of $p75^{c\text{-}rel}$ or a new avian member of the Rel family of transcription factors. $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$ differ at 10 V8 protease cleavage sites, and $p75^{c\text{-}rel}$ has 10 additional cleavage sites in its carboxy terminus which are not found in $pp59^{v\text{-}rel}$. $pp59^{v\text{-}rel}$ also has three small deletions compared with $p75^{c\text{-}rel}$ (59). These mutations explain the differences seen in the V8 maps when $p75^{c\text{-}rel}$ and $pp59^{v\text{-}rel}$ are compared.

If $p75^{c\text{-}rel}$ were truncated at the carboxy terminus (either by splicing or by proteolytic processing), then it could conceivably give rise to the proteolytic map observed for p64. Furthermore, a truncation of $p75^{c\text{-}rel}$ would explain how the protein gains access to the nucleus in normal avian cells, since it is known that truncated c-Rel expressed in cells is able to translocate to the nucleus (15, 26). To determine whether a c-Rel protein with a small truncation at the carboxy terminus would give rise to the partial proteolytic map observed for p64, sequences encoding the 40 carboxy-terminal amino acids of $p75^{c\text{-}rel}$ were deleted from the *c-rel* gene (36a), and the protein generated by in vitro transcription and translation of this truncated gene was subjected to partial proteolytic digestion. This protein had a molecular mass slightly lower than that of p64 (data not shown). $p75^{c\text{-}rel}$ was also transcribed and translated in vitro and digested with protease. The proteins generated in vitro were radioisotypically labeled with [^{35}S]methionine. Proteins metabolically labeled in vivo and used for proteolytic mapping were labeled with both [^{35}S]methionine and [^{35}S]cysteine, since c-Rel contains a limited number of methionine residues and is a protein of low abundance. Proteolytic maps of the truncated c-Rel protein ($\Delta p75^{c\text{-}rel}$) and $p75^{c\text{-}rel}$ translated in vitro were identical, as shown in Fig. 7C, lanes 3 and 4. Both share most fragments with $p75^{c\text{-}rel}$ derived in vivo (Fig. 7C, lane 2). The differences between the map of $p75^{c\text{-}rel}$ derived in vivo and in vitro may be explained by the difference in isotypic labeling procedure, since 11 cysteine residues in c-Rel are isotypically labeled in the samples derived in vivo but not in vitro. This demonstrates that p64 is not derived simply by carboxy-terminal deletion of $p75^{c\text{-}rel}$ and therefore represents a new member of the Rel family of transcription factors.

DISCUSSION

The *c-rel* proto-oncogene encodes a member of the Rel/NF-κB family of transcription factors. Most of these factors are able to bind to a 10-bp imperfect palindrome which is found in the enhancer or promoter elements of a number of cellular and viral genes (9, 50). The first NF-κB complex described consists of a heterodimer containing a 50-kDa protein (p50) and a 65-kDa protein (p65), both of which share extensive amino-terminal homology with c-Rel (4, 34). This DNA-binding complex is sequestered in the cytosol of lymphocytes by association with another protein, IκB (3). Activation of lymphocytes causes IκB to dissociate, presumably allowing the DNA-binding heterodimer to move to the nucleus (3, 51).

c-rel translated in vitro has been shown to bind the κB site by itself and as a heterodimer with p50 or p65 of NF-κB and is able to activate genes linked to the κB promoter element (21, 30). The ability to activate genes containing κB motifs is determined, in part, by the subunits which form the DNA-binding complex (44). Recent data have shown that c-Rel binds to a sequence in a human gamma interferon enhancer in which only the 3′ half-site is related to the consensus κB sequence (52). v-Rel also binds κB sites but generally suppresses transcription from promoters containing these elements (6, 30).

Experiments were performed to determine whether $p75^{c\text{-}rel}$ expression or nuclear translocation was cell cycle regulated. Although $p75^{c\text{-}rel}$ was not observed in the nucleus of avian fibroblast at any point during the cell cycle, a 64-kDa Rel-related protein was detected in the nucleus of S-phase-arrested cells. It is unlikely that p64 is the avian homolog of murine RelB or p65. Both of these murine proteins diverge from each other and from other Rel family members beyond the Rel homology domain. Since our antiserum against $p75^{c\text{-}rel}$ was generated to amino acids 347 to 579, it is unlikely that it would detect an avian homolog of either protein. A form of murine p65 has been described which has amino acids 222 to 231 deleted by alternate splicing of mRNA (41). These amino acids lie within the Rel homology domain. It is conceivable that p64 is derived from $p75^{c\text{-}rel}$ in a similar manner. This model would explain the

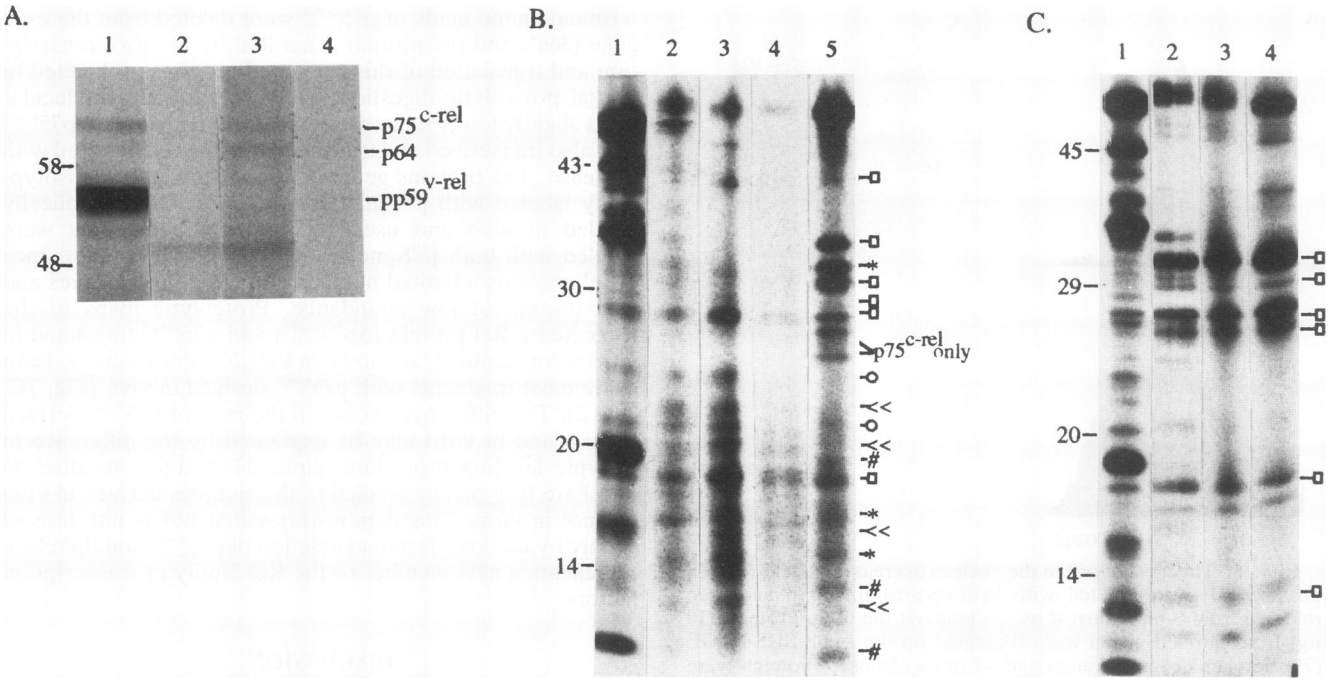

FIG. 7. p64 shares proteolytic fragments with $pp59^{v\text{-}rel}$ and $p75^{c\text{-}rel}$. (A) SDS-PAGE (7% acrylamide) of immunoprecipitations done with normal rabbit serum or antiserum against $p75^{c\text{-}rel}$ from whole-cell or crude nuclear lysates of REV-T-transformed avian lymphoid cells, or CEF arrested in S phase which were metabolically labeled with ^{35}S-amino acids. Lane 1, whole-cell lysate (100 μg) of REV-T-transformed avian lymphoid cells with antiserum against $p75^{c\text{-}rel}$; lane 2, whole-cell lysate (100 μg) of S-phase CEF with normal rabbit serum; lane 3, whole-cell lysate (100 μg) of S-phase CEF with antiserum against $p75^{c\text{-}rel}$; lane 4, crude nuclear lysate (80 μg) of S-phase CEF with antiserum against $p75^{c\text{-}rel}$. (B) Partial proteolytic digestion performed on proteins from whole-cell lysates of REV-T-transformed avian lymphoid cells or S-phase CEF or crude nuclear fractions of S-phase CEF. Fragments were resolved by SDS-PAGE (14% acrylamide). Molecular weight markers are from Pharmacia. Lane 1, $pp59^{v\text{-}rel}$ from whole-cell lysate of REV-T-transformed avian lymphoid cells; lane 2, p64 from whole-cell lysate of REV-T-transformed avian lymphoid cells; lane 3, p64 from whole-cell lysate of S-phase CEF; lane 4, p64 from crude nuclear lysate of S-phase CEF; lane 5, $p75^{c\text{-}rel}$ from whole-cell lysate of REV-T-transformed avian lymphoid cells. (C) Comparison of $p75^{c\text{-}rel}$ and $\Delta p75^{c\text{-}rel}$. Fragments were resolved by SDS-PAGE (14% acrylamide). Molecular weight markers are from Sigma and include added trypsin inhibitor and alpha lactalbumin. Lane 1, $pp59^{v\text{-}rel}$ from whole-cell lysate of REV-T-transformed avian lymphoid cells; lane 2, $p75^{c\text{-}rel}$ from whole-cell lysate of REV-T-transformed avian lymphoid cells; lane 3, $p75^{c\text{-}rel}$ translated in vitro; lane 4, $\Delta p75^{c\text{-}rel}$ translated in vitro. Symbols: □, shared by all proteins; *, shared by $p75^{c\text{-}rel}$ and p64; ○, shared by $pp59^{v\text{-}rel}$ and p64; <<, found only in p64; #, shared by v-Rel and c-Rel but not p64.

reactivity of the antiserum against $p75^{c\text{-}rel}$ and could perhaps explain the results obtained by partial proteolytic digestion of p64.

The mRNA transcript encoding $p75^{c\text{-}rel}$ is 4.0 kb (17, 39). Another transcript of 2.6 kb which is large enough to encode $p75^{c\text{-}rel}$ has been detected in the ovaries of hens and also at reduced levels in hematopoietic cells (15, 17, 39). The nature of this transcript has not been determined. We have been unable to detect a 2.6-kb transcript in avian fibroblasts. p64 could be derived by deletion of 120 bp from the 4.0-kb transcript. This alternate transcript may not be resolved by Northern (RNA) analysis. We are in the process of purifying p64 in sufficient quantity to allow peptide sequencing. This will allow design of degenerate oligonucleotides to use in cloning of the gene encoding p64.

The Fos and Jun families of transcription factors, as well as Myc and its heterodimerization partner Max, have been implicated in regulation of the cell cycle (23, 28, 36). Many different Fos-Jun complexes composed of different family members form during the G_0-to-G_1 transition and during asynchronous growth (36). The complexes which form depend on the levels of the subunits. Similarly, whether Max forms homodimers or heterodimers with Myc depends on the level of Myc available, and Myc levels rise after serum stimulation of quiescent cells (8). Although this is the first time c-Rel has been studied during the cell cycle, NF-κB has been studied during the G_0-to-G_1 transition following serum or growth factor stimulation of quiescent 3T3 cells (5, 43). NF-κB DNA-binding activity was detected shortly after stimulation but disappears near the end of G_1 and is not found in proliferating cells. This suggests that this activity is required for the G_0-to-G_1 transition. Another NF-κB-like activity was detected after stimulation but persists in proliferating cells, suggesting that different Rel/NF-κB family members are required for continuation of the cell cycle. c-*rel* is induced after serum stimulation of quiescent fibroblasts, as are the *fos* and *myc* genes, suggesting that c-Rel could also be involved in the G_0-to-G_1 transition (1, 12, 45). This study demonstrated that neither $p75^{c\text{-}rel}$ expression nor nuclear translocation is cell cycle regulated in avian fibroblasts. Perhaps $p75^{c\text{-}rel}$ is translocated during the G_0-to-G_1 transition and therefore would not have been detected in this study. We have demonstrated that a 64-kDa Rel-related protein is located in the nucleus of CEF during the S phase of the cell cycle. This observation together with the recent studies indicating that various NF-κB-like complexes are present during the G_0-to-G_1 transcription and asynchronous growth suggests that different members of the Rel/NF-κB family are regulated differentially during the cell cycle.

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