*Proc. Natl. Acad. Sci. USA*
Vol. 82, pp. 3262–3266, May 1985
Cell Biology

# Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: Subdivision of S phase

(nonhistone protein/proliferating cell nuclear antigen antibody/immunofluorescence/vertebrates/cell proliferation pathway)

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*Communicated by Diter von Wettstein, January 2, 1985*

ABSTRACT **Immunofluorescence analysis of synchronously growing transformed human amnion cells (AMA) using autoantibodies specific for cyclin has revealed dramatic changes in the nuclear distribution of this protein during the S phase of the cell cycle. Cells in $G_1$, $G_2$, and mitosis exhibit weak staining with the antibody, while S-phase cells show variable patterns of staining in terms of both intensity and distribution of the antigen. Early in S phase, cyclin is localized throughout the nucleoplasm with the exception of the nucleoli. A similar, but stronger, staining pattern is observed as the cells progress through the S phase. At a later stage, before maximum DNA synthesis, cyclin redistributes to reveal a punctated pattern with foci of staining throughout the nucleus. This pattern precedes a major change in the distribution of this protein, which is then detected in the nucleolus. At this stage, DNA synthesis is at or near a maximum. Thereafter, there are further changes in the distribution of this protein, with the pattern becoming punctated and of decreasing intensity. All these staining patterns have also been detected in asynchronously growing normal human amnion cells (AF type), suggesting that the distribution of this protein is not a consequence of transformation. Analysis of cultured cells from several vertebrate species also revealed similar staining patterns. These results are consistent with the idea that cyclin is a central component of the pathway(s) leading to DNA replication and cell division.**

Understanding of the molecular mechanisms underlying malignant transformation and cancer will be assisted by the identification of cellular proteins whose activity may be involved in the control of cell proliferation in normal cells (see refs. 1–3 and refs. therein). The acidic nuclear polypeptide cyclin ($M_r$, 36,000; for position of cyclin in two-dimensional gels, see the HeLa protein catalogue, refs. 4–6) is potentially such a candidate, as the levels of this protein are modulated during the cell cycle (increase in S phase; see ref. 7) and correlate directly with the proliferative state of normal cells. Cyclin is present in very small amounts in normal nondividing cells (senescent and quiescent cells included) and tissues, but it is synthesized by proliferating cells of both normal and transformed origin, including tumors (1, 7–25). Many of the properties of cyclin are also shared by the proliferating cell nuclear antigen (PCNA; 26–29), a human protein that has recently been shown to be identical with cyclin (21).

In this communication, we report a detailed immunofluorescence study of the distribution of cyclin (PCNA) during the cell cycle of transformed human amnion cells (AMA) by using human PCNA autoantibodies that react specifically with this protein (21). The results confirm and extend previous gel electrophoretic (7) and immunofluorescence studies (27) that showed cyclin is preferentially synthesized during S phase. Furthermore, they reveal dramatic changes in its distribution within S phase, a fact that may reflect important changes in the functional state of this protein. Taken together, these observations strengthen the idea that cyclin is a central component of the pathway that controls cell proliferation and that its activity may be related to events leading to DNA replication and cell division (refs. 1, 17, 18, and 19, and refs. therein).

## MATERIALS AND METHODS

**Cells.** All cell types used in this study were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DME medium) containing 10% fetal calf serum and antibiotics (100 units of penicillin per ml; 50 μg of streptomycin per ml). The simian virus 40-transformed human keratinocytes (SVK14) (30) were kindly provided by E. B. Lane (Imperial Cancer Research Laboratories, London). Cell cultures from various vertebrate species were a gift of J. Forchhammer (Fibiger Laboratory, Copenhagen). The normal human amnion cells were a gift of the Department of Human Genetics of this University.

**Other Procedures.** The procedures for obtaining synchronized cells by mitotic shake-off (7), labeling with [$^3$H]thymidine ([*methyl*-$^3$H]thymidine; 2 μCi/ml; 1 Ci = 37 GBq; 30 min) (14) and indirect immunofluorescence (31) have been described elsewhere. PCNA autoantibodies specific for cyclin (S17 batch) were a kind gift of M. B. Mathews (Cold Spring Harbor, New York) and R. M. Bernstein (Royal Postgraduate Medical School, London).

## RESULTS

**Immunofluorescence Localization of Cyclin in Asynchronous Transformed Human Amnion Cells.** Fig. 1*b* shows an indirect immunofluorescence picture of asynchronous transformed human amnion cells permeated and fixed with methanol and incubated with PCNA antibodies that react specifically with cyclin (21). Approximately 42% of the total cell population reacts strongly with the antibody (compare Fig. 1 *a* and *b*) to reveal a variable nuclear staining in terms of both intensity and distribution of the antigen (Fig. 1 *b* and *c*). Very little cytoplasmic staining can be observed. Some nuclear patterns of cyclin staining are indicated with arrows in Fig. 1*c*, and a description of these patterns as well as their putative sequence of appearance during the cell cycle is given below. Evidence suggesting that the differential nuclear staining observed with PCNA antibodies is due to cell-cycle variations and not to permeation or fixation artifacts has been obtained by double immunofluorescence using PCNA anti-

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Abbreviation: PCNA, proliferating cell nuclear antigen.

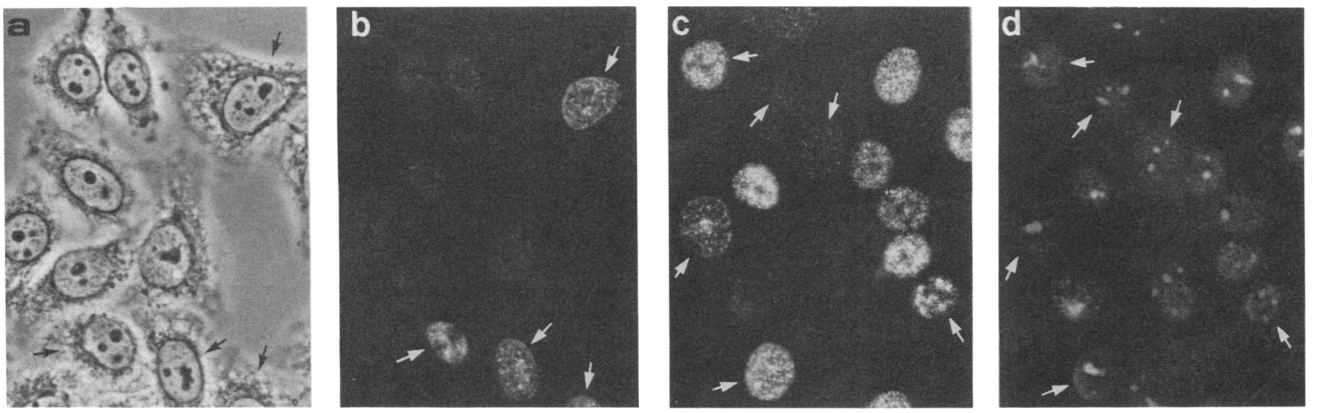


FIG. 1. Nuclear localization of cyclin in asynchronous transformed human amnion cells. (*a* and *b*) Phase contrast micrograph (*a*) and epifluorescence (*b*; PCNA antibodies) of the same field of cells treated with methanol prior to immunofluorescence. (*c* and *d*) Double immunofluorescence of methanol-treated cells reacted with PCNA antibodies (*c*) and a mouse monclonal antibody that reacts with the nucleus of all interphase cells (*d*). (×300.)

bodies (Fig. 1*c*) and a monoclonal antibody that stains the nucleus, including nucleoli, of all interphase human amnion cells (Fig. 1*d*).

**Putative Sequence of Appearance of the Various Cyclin Staining Patterns Throughout the Cell Cycle of Transformed Human Amnion Cells.** To determine the sequence of appearance of the different cyclin-staining patterns throughout the cell cycle, synchronized mitotic human amnion cells obtained by mitotic shake-off were plated on glass coverslips and analyzed at various times for [$^3$H]thymidine incorporation and indirect immunofluorescence by using PCNA antibodies (Fig. 2). The results showed a close correlation between percentage of cells exhibiting DNA synthesis and positive cyclin staining, suggesting that only S-phase cells stain strongly with the antibody. Similar observations have been reported by Takasaki *et al.* (27) in human lymphoid cells.

A putative sequence of cyclin-staining patterns deduced from observations of sister transformed human amnion cells is presented in Fig. 3. It should be stressed that the transition between the different staining patterns does not take place simultaneously in all cells and that, in most cases, the assignments have been aided by the observation of slightly asynchronous sister cells as well as of multinucleated cells showing slightly asynchronous nuclear staining patterns. During $G_1$ phase (1–6.5 hr after plating; see also Fig. 2), cyclin staining is weak but characteristic, and it is mainly confined to defined nuclear structures of unknown origin (Fig. 3*a*);

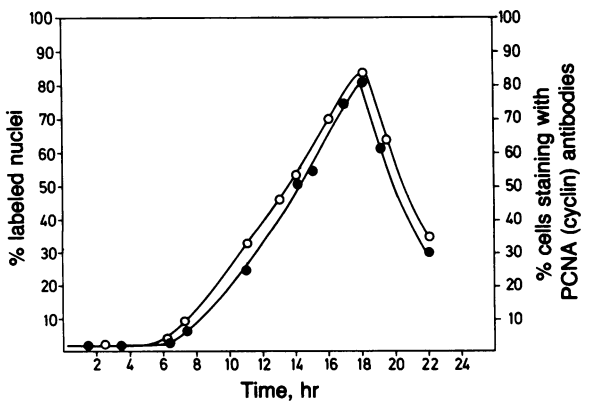


FIG. 2. [$^3$H]Thymidine incorporation (●) and cyclin staining (○) in synchronous transformed human amnion cells. Cells (grown in coverslips) withdrawn at various times after plating mitotic cells were labeled with [*methyl*-$^3$H]thymidine (30 min; 2 μCi/ml) and prepared for immunofluorescence as described (7, 31).

7–7.5 hr after plating (Fig. 2; beginning of S phase), the first cells showing increased cyclin staining are observed (Fig. 3*b*). In these cells, cyclin (granular pattern) is found throughout the nucleus with the exception of the nucleoli (Fig. 3*b*), suggesting that it is located mainly within the nucleus and not confined to the nuclear envelope. The absence of nucleolar staining has been demonstrated by comparing phase-contrast and immunofluorescence micrographs of the same field of cells (see also Fig. 1 *a* and *b*) as well as by double immunofluorescence using a monoclonal antibody that stains these structures (see also Fig. 1 *c* and *d*). A similar, although stronger, staining pattern is observed as the cells progress through S phase (Fig. 3 *c* and *d*). At a later stage, before maximum DNA synthesis (13–15 hr after plating, S-phase; see also Fig. 2), cyclin redistributes to reveal a punctated pattern with foci of staining throughout the nucleus (Fig. 3*e*). This pattern precedes a major redistribution of cyclin, which is then detected in globular structures that correspond to the nucleolus (Fig. 3*f*). In some cases, it is also possible to detect cyclin staining in distinct foci located close to the nuclear membrane (not shown, but see Fig. 3*g*). At this stage, the number of cells showing [$^3$H]thymidine incorporation and cyclin staining is at or near a maximum (17–18 hr in Fig. 2). Thereafter, there are further changes in the distribution of cyclin with the pattern becoming punctated (Fig. 3*g*) and of decreasing intensity (Fig. 3 *h* and *i*). Cells in $G_2$ phase (Fig. 3*j*) and mitosis (Fig. 3*k*; there is no chromosome staining) show only very weak staining.

Independent support for the putative sequence of patterns presented in Fig. 3 has been obtained by the analysis of asynchronous transformed human amnion cells labeled with [$^3$H]thymidine (Fig. 4*a*) prior to immunofluorescence with PCNA antibodies (Fig. 4*b*). Some corresponding patterns to those shown in Fig. 3 are indicated with the same letters in Fig. 4 *a* and *b*. In general, we find a good correlation between the number of grains present per cell and the pattern of cyclin staining.

Preliminary immunofluorescence studies of early S-phase cells from human cell lines such as SVK14 (Fig. 5*a*), A431 (carcinoma of the vulva; not shown), and HeLa (cervical adenocarcinoma, not shown) have revealed that the nucleoplasmic staining pattern exhibiting nucleolar exclusion (see also Fig. 3 *b*, *c*, and *d*) is also the first to be detected in these cells. Asynchronous cultures of these cell lines (SVK14, Fig. 5*b*; A431, Fig. 5*c*; HeLa, not shown) exhibit all the nuclear staining patterns observed in transformed human amnion cells. Moreover, these patterns are also detected in normal human amnion cells (AF type, Fig. 5*d*), suggesting

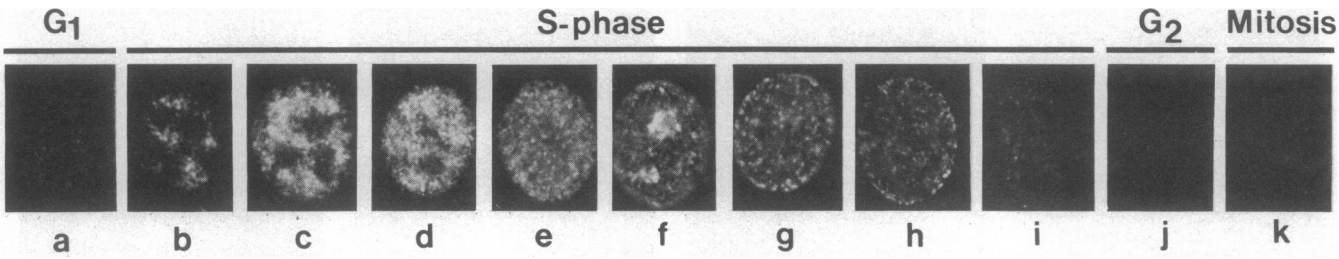


FIG. 3. Putative sequence of cyclin staining patterns during the cell cycle of transformed human amnion cells. The transition between the different staining patterns does not take place simultaneously in all cells. In most cases, the assignments were aided by the observation of slightly asynchronous sister cells as well as of multinucleated cells showing slightly asynchronous nuclear staining patterns. (×750.)

that the distribution of cyclin in transformed cells is not a consequence of transformation.

**Widespread Occurrence of Cyclin in Cultured Cells of Vertebrate Species.** Indirect immunofluorescence analysis of normal and transformed asynchronous cultured cells from various vertebrate species, using PCNA antibodies (21), confirmed previous two-dimensional gel electrophoretic studies (1, 9, 10, 12, 21, 23, 32), showing that this protein is present in hamster (CHO, not shown), mouse (3T3B, Fig. 5*e*), potoroo (PTK2, not shown), and rat cells (NRK, not shown). Cultured cells from vertebrate species not previously studied by two-dimensional gel electrophoresis that show positive nuclear staining with PCNA antibodies include aves (chicken fibroblasts, not shown), bat (lung, CCL88, Fig. 5*f*), dog (dog thymus, not shown), goat (sinovial membrane, growing, Fig. 5*g*; confluent, Fig. 5*h*), mink (lung, CCL64, Fig. 5*i*), monkey (TC7, not shown), pisces (blue gill, CCL91, not shown), rabbit (cornea, CCL60, not shown), and sheep (choroidal plexus membrane, Fig. 5*j*). In all cases, nuclear staining patterns similar to those exhibited by transformed human amnion cells are observed in these cells.

## DISCUSSION

The results presented in this article raise important questions concerning the mechanism(s) by which cyclin migrates within defined nuclear compartments during the cell cycle, particularly in S phase. The changes in the distribution of this protein are dramatic, and they are not restricted to transformed cells, a fact that underlines its putative role in normal cell proliferation in vertebrates. There are at least two likely possibilities that may explain the differential nuclear distribution of cyclin during S phase: (*i*) a direct migration of this protein or (*ii*) migration of a macromolecule(s) to which cyclin is associated. Conformational changes leading to masking/demasking of antigenic determinants should also be considered. At present, however, we cannot rule out the possibility that the observed changes in nuclear staining reflect changes in antigenicity only.

Our results concerning the preferential staining of S-phase cells with PCNA antibodies agree in general with those reported by Takasaki *et al.* (27) in human lymphoid cells. They differ, however, in the fact that they have detected nucleolar staining much earlier in S phase (late $G_1$/early S phase). At present, we do not know the reason for this difference, although it could well be related to variations in cell types.

A comment should be made concerning an apparent discrepancy between the levels of cyclin (PCNA) as determined by gel electrophoretic studies and immunofluorescence using PCNA antibodies. Significant synthesis of cyclin has been observed during $G_1$ phase and mitosis (7, 33), while

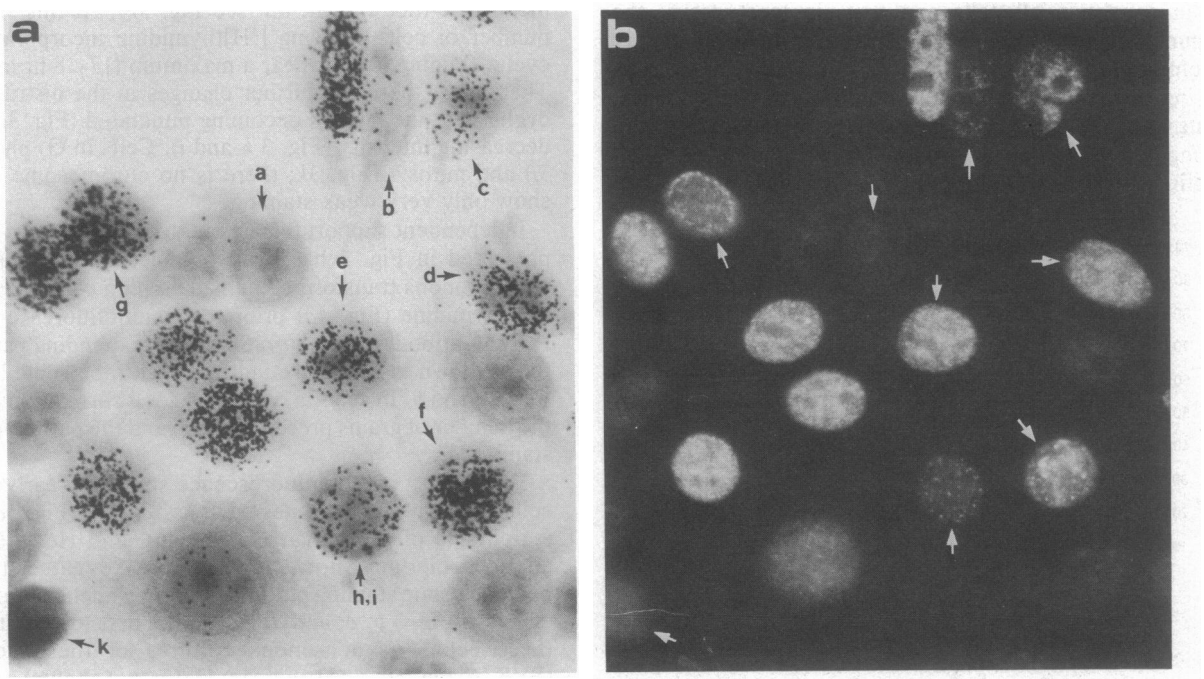


FIG. 4. Autoradiography (*a*; [$^3$H]thymidine incorporation) and epifluorescence (*b*; PCNA antibodies) of the same field of asynchronous transformed human amnion cells. Cells grown in coverslips were labeled with [*methyl*-$^3$H]thymidine (30 min; 2 μCi/ml), fixed with methanol, and reacted with PCNA antibodies. Immunofluorescence pictures were taken prior to autoradiography. (×450.)

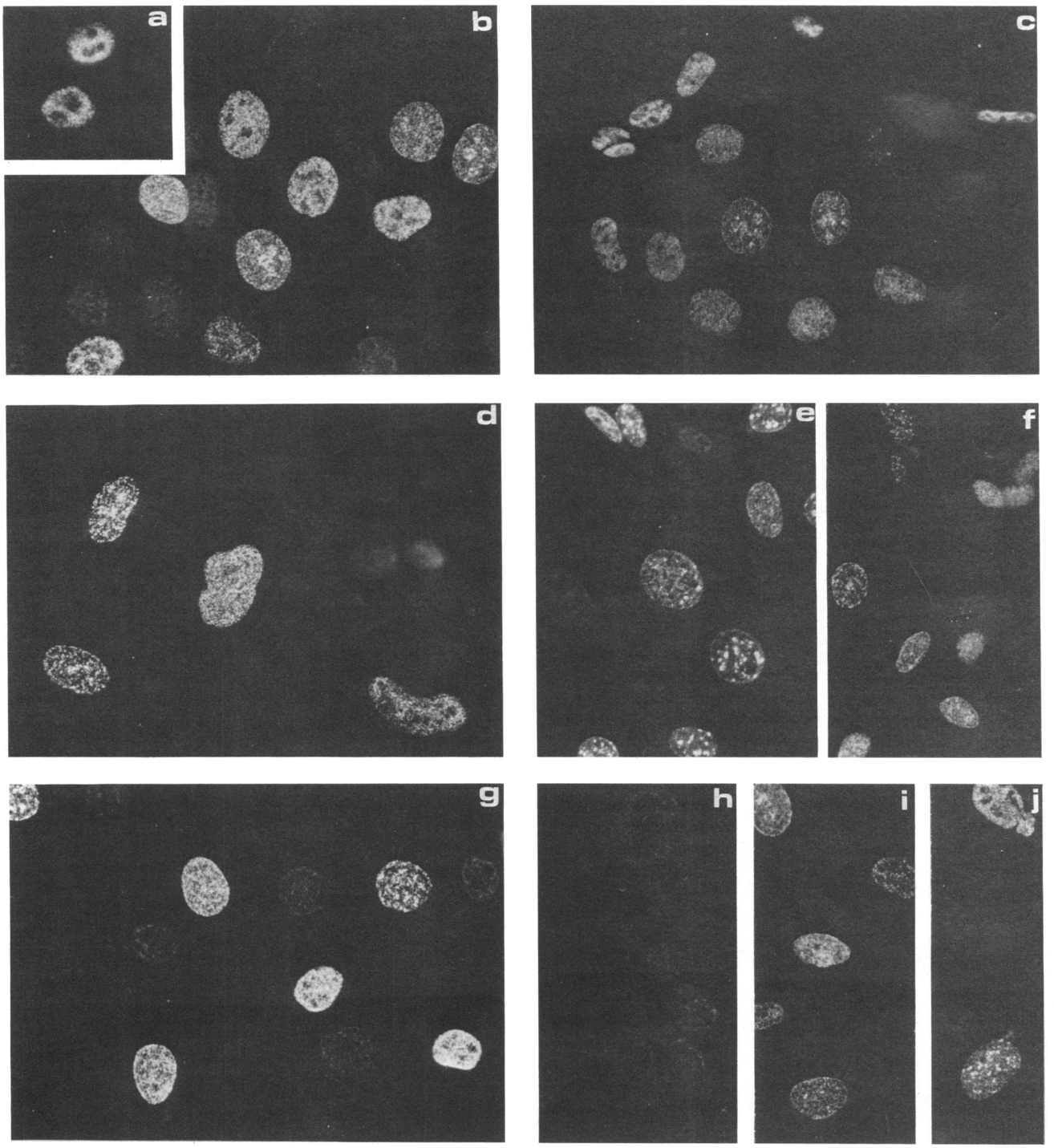


FIG. 5. Nuclear localization of cyclin in cultured cells from vertebrate species. Cells grown in coverslips were treated with methanol prior to immunofluorescence. (*a*) Synchronous cells; (*b–i*) asynchronous cells. (*a*) Early S-phase SVK14 cells, (*b*) SVK14, (*c*) A431, (*d*) normal human amnion cells, AF type, (*e*) mouse 3T3B, (*f*) bat, (*g*) goat sinovial membrane (cycling cells), (*h*) goat sinovial membrane (confluent), (*i*) mink lung, and (*j*) sheep choroidal plexus membrane. (×388.)

only comparatively weak staining is detected with the antibody. Therefore, it is likely that the antibody may not recognize all forms of cyclin in fixed cells. In fact, enucleated transformed human amnion cells (cytoplasts) known to synthesize cyclin (unpublished results) give only weak staining with PCNA antibodies.

Finally, we would like to emphasize that autoradiographic studies of Chinese hamster ovary cells labeled with [$^3$H]thymidine have revealed various topographical patterns of DNA synthesis (34, 35) that are strikingly similar to those observed with PCNA antibodies. For example, the absence of nucleolar labeling seems to be characteristic of early S-phase cells, while nucleolar labeling has been observed late in S phase (34, 35). These observations, together with recent experiments suggesting that the distribution of cyclin depends in part on the status of DNA replication (unpublished observations; see also ref. 36), are consistent with the idea that cyclin is a central component of the pathway(s) leading to cell division and that its activity may be associated with events involved in DNA replication (1, 17, 18).

We thank R. Garrett for reading the manuscript and O. Jensen for photography. This work was supported by grants from EURATOM, The Danish Medical and Natural Science Research Councils, the

Danish Cancer Foundation, the Aarhus University Research Fund, and Novo Industri.


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