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Cell Cycle Control of the Human HSP70 Gene: Implications for the Role of a Cellular E1A-Like Function

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The gene encoding the human 70-kilodalton heat shock protein (HSP70) is subject to activation by the adenovirus E1A gene product and appears to be regulated in the absence of heat shock by a cellular activity similar to E1A. Given the relation of E1A to alteration of growth control, we have investigated the expression of the HSP70 gene during the cell cycle. Assay of mRNA levels after release from a thymidine-aphidicolin block revealed a 20-fold increase in mRNA abundance, reaching a peak level in the post-S-phase period. Upon reaching this peak level, the abundance of the mRNA then declined as the cells entered the next cycle. Control of the abundance of the mRNA during the cell cycle appeared to be primarily at the level of transcription as measured in nuclear runoff assays. Very similar results were obtained by analyzing the expression of the HSP70 gene in the adenovirus-transformed 293 cell line. Furthermore, the E1A gene was also found to be cell cycle regulated; the activation and peak level of the E1A mRNA occurred at an earlier time than those of the heat shock mRNA, consistent with, but not proof of, the hypothesis that E1A is responsible for the cell cycle control of the HSP70 expression. We therefore suggest that the E1A-like cellular activity may govern certain aspects of cell cycle transcription.

The control of transcription initiation is of central importance to diverse aspects of biology including cellular differentiation, cellular responses to hormones, and oncogenesis. A great deal of information has accumulated over the past several years concerning genes that are subject to transcription control (for reviews, see references 6, 31). Sequences that are important for such regulation have been defined in a number of genes (for instance, see reference 12) as well as have factors that interact with such sequences (8, 9, 33). A final understanding of a system of gene control must also include the mechanisms that regulate the action of such factors, whether that means controlling the specific activity or the abundance of the factors within a particular cell. To date, the only genes identified that function as positive regulators of transcription in mammalian cells are the adenovirus E1A gene and the herpesvirus immediate early gene. During normal lytic infections of these viruses, these genes are responsible for the stimulation of transcription of the set of viral genes that are expressed before DNA replication (2, 7, 21, 29, 35, 42).

Several lines of evidence suggest that these viral regulatory genes may represent general activators of transcription and possibly close approximations of cellular regulatory genes. First, the action of these genes is not restricted to the homologous viral genes that they normally activate. The herpesvirus immediate early gene can activate early adenovirus genes; in fact, the herpesvirus-mediated transcriptional activation is considerably more efficient than the E1A-mediated activation (10, 19). Second, we have previously shown that a cellular gene encoding the 70-kilodalton heat shock protein (HSP70) is subject to transcriptional activation by the adenovirus E1A gene product (22, 30). This observation had led to the suggestion that the HSP70 gene may be regulated in growing cells by an activity similar to E1A. Furthermore, a number of cellular genes, not related to adenovirus, are subject to stimulation by the E1A gene when introduced into cells by DNA-mediated transfection (11, 14, 41). Thus, it appears that the E1A gene has the ability to effect a rather broad activation of transcription within the cell. Third, by analyzing the expression of the HSP70 gene in various cells, it was found that cells expressing a high level of heat shock mRNA also could partially provide E1A function for early adenovirus transcription (20). This result suggests the presence of a cellular activity similar in nature to E1A.

What might be the role of such an E1A-like activity in the normal growing cell? Since the adenovirus E1A gene has the ability to immortalize cells in culture and since we have previously observed that cells possessing a high E1A-like activity were usually rapidly growing (20), we asked whether the control of HSP70 expression may be a growth-related phenomenon. Specifically, we have investigated the expression of the HSP70 gene during the cell cycle. Indeed, we find that the HSP70 gene is cell cycle regulated in HeLa cells and that this regulation is largely at the level of transcription.

MATERIALS AND METHODS

Cells. HeLa cells and 293 cells were grown in spinner culture in Joklik-modified minimal essential medium containing 5% fetal calf serum. Cells were synchronized as described previously (15).

RNA analysis. The preparation of RNA and analysis by northern blotting were performed as described previously (22).

Isolated nucleus transcription. The preparation of nuclei and the preparation of pulse-labeled RNA were as previously described (4).

RESULTS

Cell cycle control of HSP70 mRNA. We have used spinner cultures of HeLa cells for these experiments because of previous studies that have shown that the HSP70 gene is expressed at relatively high levels in growing HeLa cells (20). HeLa cells were synchronized at the G1/S boundary,

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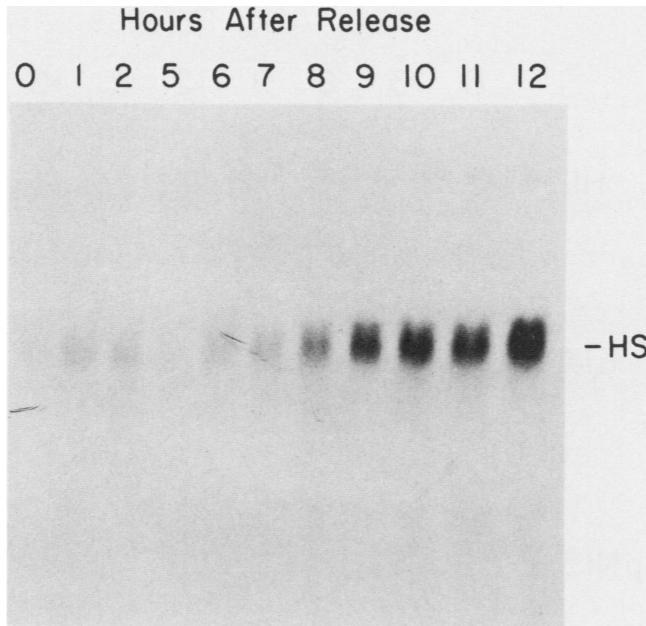

FIG. 1. Kinetics of accumulation of HSP70 mRNA in synchronized HeLa cells. HeLa cells were synchronized with a thymidine-aphidicolin block and then released into S phase by repeated washing and resuspension in fresh media. At the indicated times, 5 $\times 10^7$ cells were harvested and RNA was prepared. Equal amounts of RNA (10 μg) were fractionated into an agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled HSP70 cDNA probe (pHSG).

using a thymidine block followed by an aphidicolin block (15, 34). To measure the expression of the HSP70 gene, we have used a cDNA copy of the human HSP70 mRNA (22). Such a cDNA clone has been shown to detect a 2.7-kilobase mRNA that increases in abundance in HeLa cells after either a heat shock or an adenovirus infection (22). Synchronized HeLa cells were released from the aphidicolin block and RNA was then prepared at various times. A Northern analysis of such RNA is shown in Fig. 1. In the blocked cells and throughout most of S phase, heat shock mRNA levels were relatively low, but then increased substantially (approximately 20-fold) towards the end of S phase. In this experiment, S phase occurred between 1 and 8 h after release as defined by [^{3}H]thymidine incorporation (data not shown). To determine if there was actually a cycling control of heat shock mRNA abundance, RNA was analyzed in another experiment with time points extending to 17 h post-release, thus through a complete cell cycle. The level of the heat shock mRNA peaked at 12 h post-release and then declined before the next cell cycle (Fig. 2). Also shown for comparison in Fig. 2 is the pattern of histone H4 mRNA abundance levels. From these results, it is clear that the HSP70 mRNA is expressed during a restricted period of the cell cycle and that the kinetics of HSP70 accumulation are distinct from the S-phase kinetics of histone mRNA.

Transcriptional regulation of HSP70 during cell cycle. It has been documented that the E1A-mediated induction of the heat shock gene occurs primarily at the level of transcription (22), although E1A may effect post-transcriptional control on viral gene expression as well (23). Thus, if the cell cycle regulation of the heat shock gene is in fact analogous to E1A control, we would expect that there would be transcriptional changes occurring during the cell cycle. For these experiments, we have used the technique of nuclear runoff transcription assays in isolated nuclei to measure rates of transcription. Nuclei were prepared at various times after release from a thymidine-aphidicolin block and incubated with [^{32}P]UTP, and the labeled nuclear RNA was isolated and hybridized to plasmid DNAs immobilized on nitrocellulose. The intensity of the hybridization signal constitutes a measure of the rate of transcription for that particular gene. We found that the heat shock gene clearly responded at the level of transcription (Fig. 3); in the cells blocked at G1/S, transcription of the gene was barely detectable. Upon release, there was a large increase in transcription rate that peaked at 8 h after release from the block, thus somewhat preceding the time of maximal mRNA accumulation. In fact, there was an approximately 20-fold increase in transcription of the gene during the cell cycle. Therefore, we can conclude that whatever activity is responsible for the control of the heat shock gene during the cell cycle does so primarily at the

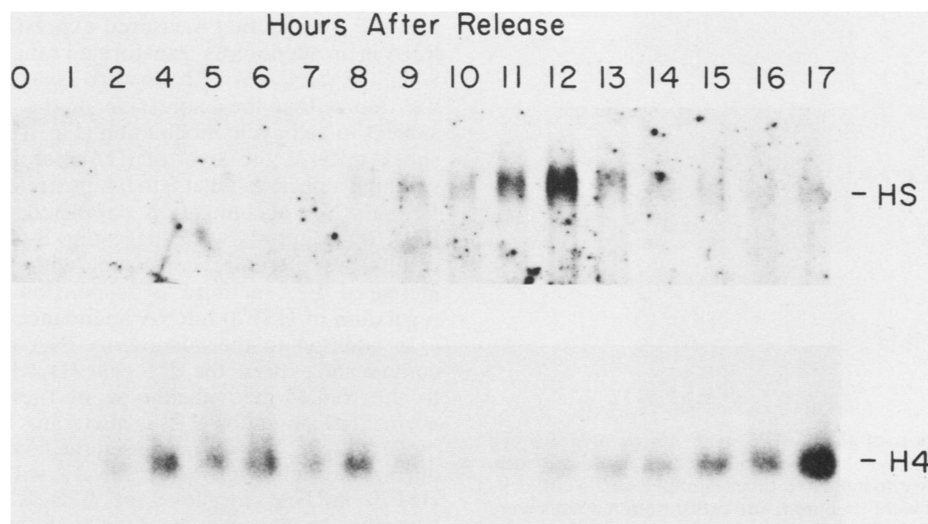

FIG. 2. Kinetics of accumulation of HSP70 mRNA during a HeLa cell cycle. RNA was prepared and analyzed as indicated in the legend to Fig. 1. The same set of RNAs was also probed with ^{32}P-labeled pHu4A, a human histone H4-specific clone (16).

level of transcription (we cannot rule out additional posttranscriptional effects) and that this most likely represents the controlled fluctuation of a specific transcriptional regulatory activity.

Cell cycle control of HSP70 mRNA in 293 cells. Our observation that the heat shock gene is cell cycle regulated in HeLa cells suggests that whatever gene is controlling heat shock gene expression may also be cell cycle regulated. At this time, however, we have no direct assay for the proposed E1A homolog in HeLa cells, and we cannot directly demonstrate the involvement of such a gene in cell cycle-related events. As an alternative, we have investigated the expression of the HSP70 gene in the adenovirus-transformed 293 cell line (13). Heat shock mRNA is expressed at very high levels in 293 cells (22), and this expression is governed at the level of transcription when compared with other cells in which expression is low (H.-T. Kao and J. R. Nevins, unpublished data). We have suggested that this high basal level of heat shock expression is mediated by the action of E1A based on the observation that E1A induces the HSP70 gene during a lytic viral infection (22, 30). Using the 293 cells, we can thus ask if the heat shock gene is cell cycle regulated and, if so, is the E1A expression also regulated in a manner consistent with its potential control of the HSP70 gene.

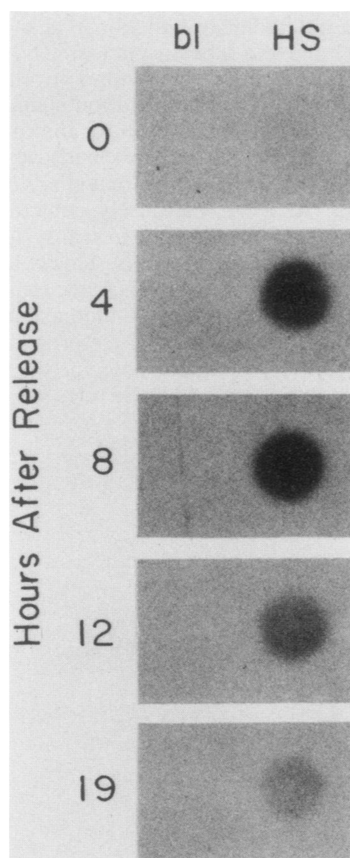

FIG. 3. Transcription of the HSP70 gene during a HeLa cell cycle. HeLa cells were synchronized and released as described in the legend to Fig. 1. At the indicated times, samples of 5×10^7 cells were removed, nuclei were prepared, and transcription assays were carried out as described in the text. The labeled nuclear RNA was hybridized to filters bearing pHSG (HS) or pBR322 (bl). After hybridization, filters were washed and then treated with RNase.

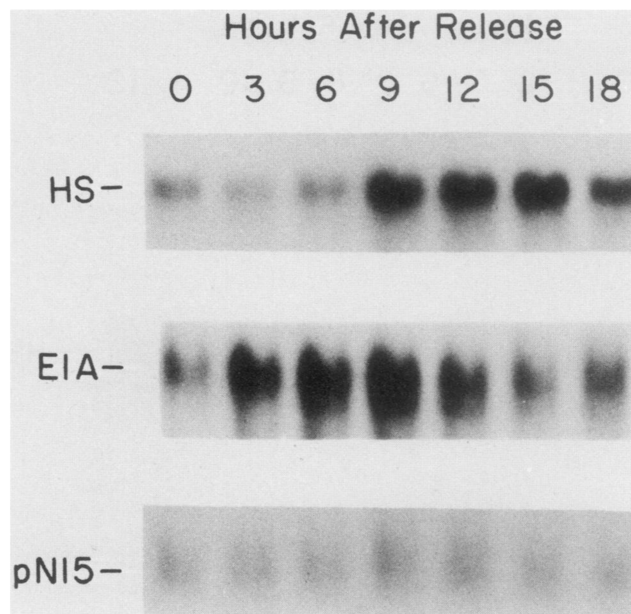

FIG. 4. Kinetics of accumulation of HSP70 and E1A RNAs during a 293 cell cycle. 293 cells were synchronized and released as described for HeLa cells in the legend to Fig. 1. Cytoplasmic RNA was prepared from cells harvested at the indicated times and HSP70 mRNA was detected as described in the legend to Fig. 1. Adenovirus E1A mRNA was detected in the same RNA samples, using an adenovirus 2 DNA clone representing genomic sequences of 0 to 4.4 map units. As a control, the same RNAs were probed with a 293 cell cDNA clone (pN15) that does not vary with the cell cycle.

293 cells were synchronized and released into S phase, and the abundance of the HSP70 mRNA was assayed at various times. A pattern of regulation of the HSP70 mRNA abundance was found in 293 cells that was similar, although not identical, to that found in HeLa cells (Fig. 4). Once again, the level of HSP70 mRNA in the blocked cells was quite low; upon release, the level rose to a peak value between 9 and 15 h postrelease. Of course the advantage of the 293 cell system is the fact that we can assay for the expression of the E1A gene, the potential inducer. That the E1A gene may be cell cycle regulated was suggested by previous studies that measured expression of virus-specific RNA in an adenovirus-transformed rat cell line (17). Indeed, when the same set of RNAs were assayed for E1A mRNA it was found that the expression of the E1A gene was also subject to cell cycle modulation (Fig. 4). In contrast to heat shock mRNA, the level of E1A rose rapdily after release reaching a peak value at 6 to 9 h postrelease. The kinetics of E1A mRNA accumulation paralleled that of histone H4 RNA (data not shown), suggesting S-phase control. These results are therefore consistent with the hypothesis that increased E1A activity is responsible for the cell cycle regulation of HSP70 mRNA abundance in 293 cells.

In addition to the adenovirus E1A gene, 293 cells also contain and express the E1B gene (1), which also contributes to the transformed phenotype of these cells. Since in a normal lytic infection E1B is also transcriptionally regulated by E1A (2, 21, 29), we measured the level of the E1B mRNA during the cell cycle (Fig. 5). As with E1A mRNA and HSP70 mRNA, the level of E1B RNA also fluctuated according to the cell cycle. One might have expected to find that the expression of E1B follows that of the HSP70 gene since both are E1A inducible in a lytic infection. However,

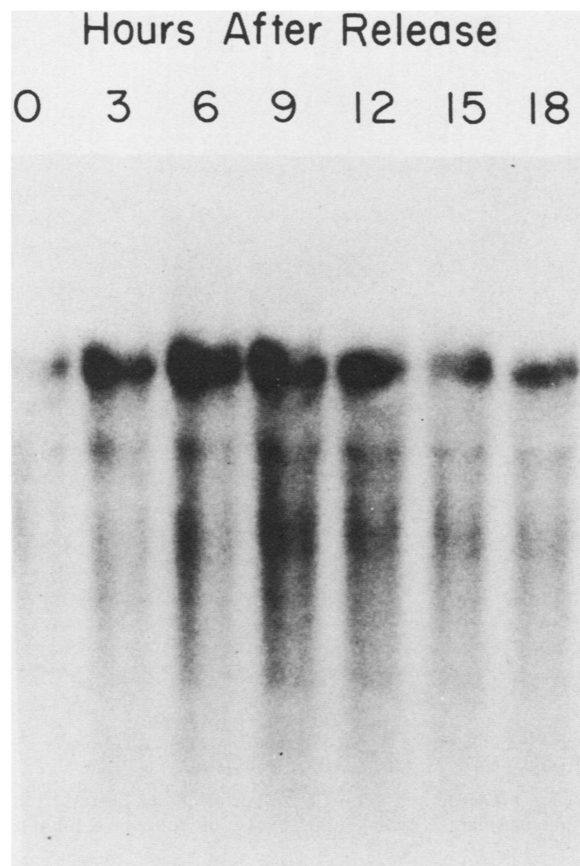

FIG. 5. Kinetics of accumulation of E1B RNA during a 293 cell cycle. The same set of RNAs analyzed in Fig. 4 was hybridized with an adenovirus 2 E1B-specific DNA clone (4.4 to 11.0 map units).

the kinetics of accumulation appeared to more closely follow that of E1A, peaking in abundance at 6 to 9 h postrelease.

Transcriptional regulation of HSP70 in 293 cells. To ascertain the level of control in the 293 cells, transcription in isolated nuclei was again measured during the cell cycle. As was the case in HeLa cells, expression of the HSP70 gene was controlled at the level of transcription (Fig. 6). The degree of control did not appear to be as dramatic in 293 cells as in HeLa cells, primarily due to a higher basal level in the cells blocked at the G1/S boundary. That is, in several separate experiments the transcription of the gene did not decline to the low level that was observed in HeLa cells. Nevertheless, the degree of change during the experiment was 6 to 10-fold, thus largely accounting for the change in mRNA levels.

In contrast, transcription of the E1A or the E1B genes in 293 cells did not change as a function of time after release from blocked cells (Fig. 7) despite the fact that the abundance of the mRNAs varied during this period by a factor of 10. Therefore, the change of abundance as measured by northern analysis must be largely due to post-transcriptional effects. Possibly the nature of the E1A and E1B integration event has altered the normal E1A-mediated regulation of E1B that one finds in a lytic viral infection.

DISCUSSION

We have previously suggested that certain cells possess an activity that controls transcription in a manner similar to the adenovirus E1A gene product (20). In this report, we suggest that such an activity is involved in cell cycle regulation of transcription. This suggestion is based on several observations, including the following. One gene under the control of an E1A-like activity, the HSP70 gene, is cell cycle regulated; furthermore, this regulation resides almost completely at the level of transcription, consistent with the mechanism of E1A-mediated induction of the HSP70 gene (22). Finally, the HSP70 gene is cell cycle regulated in 293 cells as well as in HeLa cells, and the relative kinetics of E1A and HSP70 expression in 293 cells are consistent with E1A control. We do not mean to suggest that the E1A gene product or a cellular analog of E1A might be responsible for all cell cycle transcriptional control. We do mean to suggest that this class of gene (E1A) may be involved in the control of one gene (HSP70) that is cell cycle regulated and possibly other genes as well.

Might these results also imply that the E1A-mediated activation of early adenovirus transcription is a process analogous to cell cycle control of gene expression? An involvement of adenovirus in cell cycle events has previously been suggested since adenovirus can induce cellular DNA replication in serum-starved cells (38, 40, 44). In addition, there may indeed be an involvement of the E1A gene in stimulating thymidine kinase expression, although it is not clear that this is a direct activation (3). The viral life cycle is devoted to the replication of additional viral genomes, presumably the assembly of these new genomes into some form of chromatin structure, and the packaging of this new DNA into progeny viral particles. Although only a few of the early adenovirus genes have been defined in terms of function, one must nevertheless presume that many are involved, directly or indirectly, in this process. This certainly is the case for the proteins whose function is known, including the DNA-binding protein, the DNA polymerase, and the DNA-linked terminal protein (for review, see reference 39). If one considers that a temporal program of gene expression is also required to provide components necessary for DNA replication and packaging of the cellular genome in preparation for division, then this type of analogy becomes more obvious. One might, therefore, speculate that through evolution the virus has acquired and modified certain cellular genes which are necessary for DNA replication or chromosome packaging or both. It seems possible that, along with these genes, the virus may have acquired the regulatory gene (E1A) responsible for their coordinate activation.

A role for E1A in cell cycle transcriptional control bears on the role of E1A in oncogenesis. The E1A gene has been described as an immortalizing function, conferring unlimited growth potential to an otherwise permanently quiescent cell (18, 37). One could imagine that immortalization was in part the result of providing a stimulus for transcription of those cellular genes required for cell cycle progression. Certain recent results would, however, appear to argue against such a role for E1A in transformation. The E1A transcription unit produces two mRNAs (13S and 12S) during an early lytic infection and mutant studies have shown that the product of the 13S mRNA is responsible for viral transcription activation (28, 36). Montell et al. (27) have shown that the E1A 12S product possessed little if any transcription-inducing function but that this E1A product alone could still effect partial transformation. However, Leff et al. (26) have shown by transfection assays that both the 12S product and the 13S product could induce early transcription. Furthermore, Winberg and Shenk (43) have shown, using 13S only and 12S only viruses, that the 12S product still possessed some

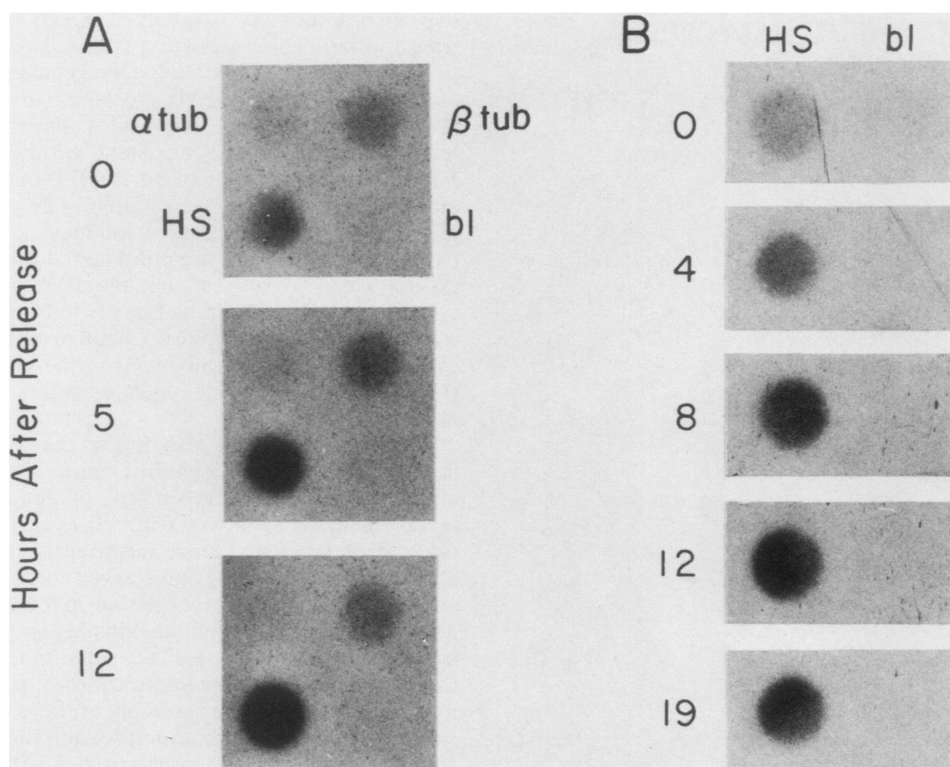

FIG. 6. Transcription of the HSP70 gene during a 293 cell cycle. Nuclei were prepared and assays were performed as described in the legend to Fig. 3. (A) and (B) represent two separate experiments. The filters in experiment A included plasmids containing the α-tubulin and β-tubulin genes.

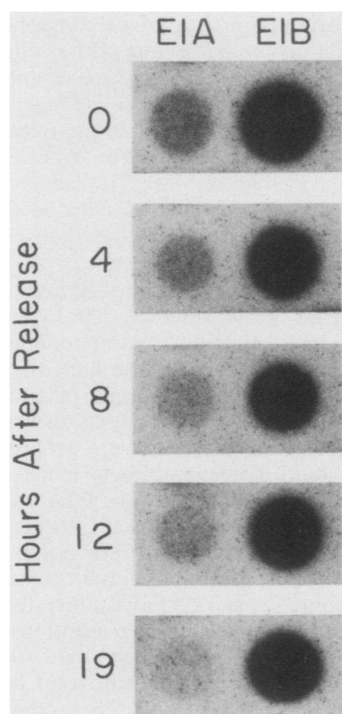

FIG. 7. Transcription of the E1A and E1B genes during a 293 cell cycle. The preparation of nuclei and the assays were as described in the legend to Fig. 3.

activity, although less than the 13S product. Certainly what is not clear is how much transcriptional induction is required for immortalization, and if the 12S E1A product still possessed some of the activity of the 13S product, as suggested by these recent studies, then this might be sufficient for stimulation of appropriate cellular genes.

What is clear from these studies, and possibly the most important point from the standpoint of regulation of gene expression, is that the HSP70 gene is transcriptionally regulated during the cell cycle. It will be important to examine this aspect of HSP70 gene expression in cells synchronized by other procedures to more clearly define the precise points of activation and suppression of this gene. However, it is quite clear that the transcriptional regulation of this gene is more profound than that found for the histone genes and certainly for any of the other known cell cycle genes. It would thus appear that the study of this gene, in terms of sequences and factors required for such regulation, should yield clear and valuable information regarding this important aspect of gene expression and cellular growth control.

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