STUDIES ON THE CONTROL OF DEVELOPMENT SYNTHESIS OF REGULATORY NUCLEOTIDES, HPN AND MS, IN MAMMALIAN CELLS IN TISSUE CULTURES

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Received 12 December 1974
Revised version received 28 February 1975

1. Introduction

Unusual highly phosphorylated substances, HPN, were first discovered to appear at the beginning of sporulation in *B. subtilis* [1]. Initially only two substances were visible in a one-dimensional thin-layer chromatogram of formic acid extracts of sporulating, but not vegetative cells. Two-dimensional PEI thin-layer chromatography revealed, however, that six nucleotides carrying more than three phosphate groups are synthesized shortly before, at, or shortly after the beginning of development [2,3]. Two of these substances are MS I (guanosine-5′-diphosphate-3′-diphosphate, ppGpp) and MS II (guanosine-5′-triphosphate-3′-diphosphate, pppGpp), which have been found originally in *E. coli* starved of a required amino acid [6]. Experiments with a normally sporulating relaxed mutant of *B. subtilis* [4], unable to accumulate MS I and II after amino acid starvation showed, that the MS nucleotides are not needed for development (differentiation).

The highly phosphorylated nucleotides HPN I, II, III, and IV, in contrast, seem to be associated with sporulation. Especially HPN IV (adenosine-5′-triphosphate-3′-triphosphate, pppAppp) and HPN III (tentative structure is uridine-5′-diphosphate-3′-diphosphate, ppUpp) are produced only, when sporulation occurs [3]. HPN I (adenosine-5′-diphosphate-3′-diphosphate, ppApp) and HPN II (adenosine-5′-triphosphate-3′-diphosphate, pppApp) are synthesized by ribosomes from sporulating but not from those of vegetative cells of *B. subtilis* [5] indicating, that these substances are also involved in the regulation of development.

Since it is possible that the basic phenomena of differentiation exhibited by *B. subtilis*, our simple model system for studying differentiation, resemble those in higher organisms, we searched for the presence of unusual nucleotides in three different types of mammalian cells in tissue culture, namely in Chinese hamster ovary cells (CHO), baby hamster kidney cells (BHK) and human lung cells (WI 38). As will be described in this communication, all three types of cells accumulate either one or more of these regulatory nucleotides.

2. Materials and methods

2.1. *Cell lines*

Chinese hamster ovary cells, CHO (Cricetulus griseus), ATCC cell repository designation CCL 61, Syrian hamster kidney cells, BHK-21 (*Mesocricetus auratus*) ATCC cell repository designation CCL 10, and human diploid lung cells, WI-38, ATCC cell repository designation CCL 75 were used in these studies.

2.2. *Media and growth conditions*

The media used for growth of the three different cell lines was Eagles basal medium No. 2 (obtained from Flow Laboratories together with the other ingredients contained in the medium) enriched with L-glutamine (200 mM), 5000 units/ml each of Penicillin and Streptomycin, with non-essential amino acids (1x) and 10% calf serum.

All cell lines, BHK, CHO, and WI-38 were obtained from the American Type Culture Collection, tested and shown to be free of mycoplasmas, bacteria, fungi, protozoa and cytopathic viruses. They were grown for 3 to 5 generations in the above described medium. Then, a monolayer of cells in a 75 cm^2 Falcon flask was trypsinized and distributed evenly either into three 75 cm^2 or nine 25 cm^2 Falcon flasks.

For labeling of the unusual nucleotides, 1 mCi $H_3[^{32}P]O_4$ (Amersham-Buchler, carrier free steril solution) was used per 25 cm^2 Falcon flask.

Growth and qualitity of cells was tested microscopically. The absence of bacterial contaminations was checked regulary by plating 5 times 0.1 ml of the culture medium on nutrient agar plates [3]. The absence of mycoplasma contaminations was shown by standard culture procedures (mycoplasma test, Flow Laboratories, Bonn, Germany) and biochemically using the uridine phosphorylase method [11]. No culture used in the experiments described below showed any traces of contaminations by bacteria or mycoplasmas.

2.3. *Determination of cell numbers*

Cell numbers were determined after trypsinization and resuspension in PBS-buffer (without calcin) with a Coulter counter.

2.4. *Extraction of low molecular weight substances*

Low molecular weight substances were extracted from ^{32}P-labeled cells grown in a 25 cm^2 Falcon flask. After removal of the medium by aspiration, the cells sticking to the surface of the flask were overlayed with 2 ml ice-cold 2 M formic acid, pH 3.4. After 15 min at 5°C, the supernatant was carefully removed from the cells, which still stick to the surface and immediately frozen until used for chromatography.

When the growth medium was tested for the presence of ^{32}P-labeled nucleotides, a sample of the medium was carefully removed without touching the surface of the flask and immediately frozen at −20°C until used for chromatography.

2.5. *Thin-layer chromatography*

Thin-layer chromatography on PEI thin-layer plates was performed as described previously [5].

3. Results

When CHO cells are grown for 48 hr at 37°C and then incubated for additional 12 hr in the presence of $H_3[^{32}P]O_4$ (see Materials and methods), unusual nucleotides are detectable in formic acid extracts of these cells (see fig.1, column 3) and the supernatant medium (column 2) after separation by one-dimensional PEI thin-layer chromatography. Two of these unusual nucleotides found between the origin and GTP [fig.1, column 1) migrate similar to MS I (fig.1, column 3 and 5) and MS II (fig.1, column 3, 5 and 7). These nucleotides have been discovered in *E. coli* after amino acid starvation previously [6].

Since we have shown [3] that inhibitors of protein synthesis can stimulate the accumulation of MS I (ppGpp) and MS II (pppGpp) in *B. subtilis*, actomycin

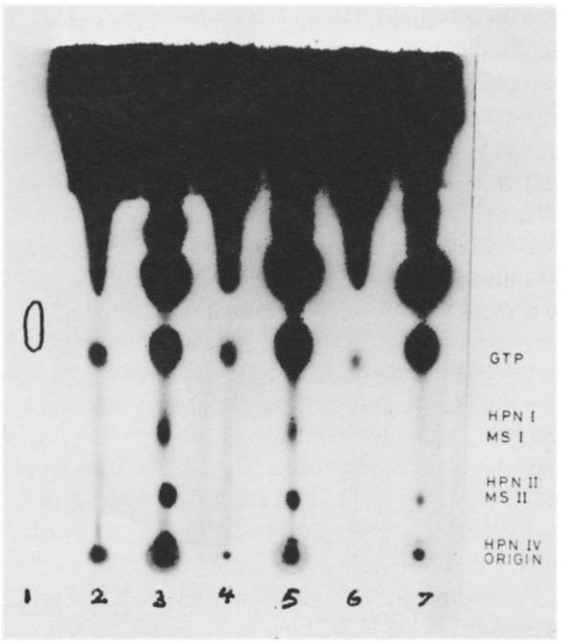

Fig.1. Unusual nucleotides in media and formic acid extracts of mammalian cells with and without treatment of actinomycin D as described in Materials and methods. 1. unlabeled GTP (Boehringer, Mannheim) 2. medium of CHO cells, 3. formic acid extract of CHO cells, 4. medium of actinomycin D treated CHO cells, 5. formic acid extract of cells as in 4., 6. medium of actinomycin D treated BHK cells, 7. formic acid extract of cells as in 6. 10 µl of each, medium and extract, was applied to PEI thin-layer chromatograms and further treated as described (see Materials and methods).

D, which inhibits replication and transcription and as a consequence also protein synthesis, was added 1 hr after the addition of $H_3[^{32}P]O_4$ to a 48 hr culture of CHO cells.

As can be seen in fig.1, the same nucleotides as before can be detected in the medium (column 4) and in cell extracts (column 5) after 2 hr of actinomycin treatment of CHO cells.

Similar results are obtained when BHK cells are treated in the same manner. As in the previous cases, the supernatant (column 6) contains less ^{32}P-labeled unusual nucleotides than a formic acid extract of cells (column 7). Remarkable, however, is the lower concentration of MS I in BHK cells as compared to MS I in formic acid extracts of CHO cells with and without actinomycin D treatment. Careful investigations of the autoradiogram shown in fig.1 revealed that the medium of these cultures contains three substances (some only weakly labeled) migrating slower than GTP on PEI thin-layer chromatograms. Two of these spots are different from the major substances found in formic acid extracts. However, close inspection of the formic acid extracts revealed that these substances, though not clearly separated from the major substances, are also present intracellulary, i.e. can be extracted by formic acid treatment (column 3, 5 and 7).

We do not know, why in these experiments some of the unusual nucleotides of formic acid extracts can also be detected in the medium, while others cannot. In some other experiments (Rhaese, Grade and Dichtelmüller, unpublished data) we did not find significant amounts of unusual nucleotides in the medium. Whether this 'leakage' of nucleotides into the medium is due to as yet unknown factors, like exhaustion of the medium etc., is presently under investigation.

All five substances (a sixth unusual nucleotide can only be detected by two-dimensional thin-layer chromatography) found in these mammalian cells have been detected previously in *B. subtilis* [2,7]. But the supernatant medium of *B. subtilis* cultures in contrast to CHO and BHK cells contains only one substance, named HPN IV, the adenosine-hexaphosphate [2,7], migrating near the origin. This difference could be due to differences in cell wall and membrane structure of bacteria and mammalian cells. Apparently, leakage of unusual nucleotides with the exception of HPN IV, which is found in *B. subtilis* only extracellularly [2], is prevented in bacteria.

Two-dimensional PEI thin-layer chromatography (see Materials and methods) and chromatographic comparison with all unusual substances found in *B. subtilis* shows that the three major substances present in formic acid extracts of CHO and BHK cells are MS I, MS II and HPN IV (see fig.1, column 3, 5 and 7). The location of these substances in a two-dimensional chromatogram has been shown by us previously [2,7]. MS I (guanosine-5′-diphosphate-3′-diphosphate, ppGpp) and MS II (guanosine-5′-triphosphate-3′-diphosphate, pppGpp) have been synthesized by ribosomes from vegetative *B. subtilis* cells [5] or *E. coli* [10]. HPN IV (adenosine-5′-triphosphate-3′-triphosphate, pppAppp) was isolated from the supernatant of sporulating *B. subtilis* cells [7].

The substances found in the supernatant medium of these cells comigrate with HPN I, HPN II and HPN IV. HPN II, which is adenosine-5′-triphosphate-3′-diphosphate, pppApp, [9] has also been synthesized by ribosomes from sporulating *B. subtilis* cells together with HPN I, adenosine-5′-diphosphate-3′-diphosphate, ppApp [5].

HPN III, which has not been clearly identified in CHO or BHK cells is probably present also but its concentration is low compared to the other nucleotides. Further investigations are necessary to varify this possibility. HPN III, which is likely to be the tetraphosphate of uridine (complete structure is not known yet) and HPN IV have been shown to be closely connected with sporulation (differentiation) in *B. subtilis* [7]. When the nucleotides present in formic acid extracts were separated by PEI thin-layer chromatography (see Materials and methods), cut out together with GTP, and the radioactivity counted in a Scintillation spectrometer (Packard-Tricarb), the following relative concentrations of these nucleotides compared to GTP are obtained (table 1).

Table 1
HPN and MS nucleotides found in CHO and BHK cells

Cell line	Actm.* treatment	Relative amount (%) of GTP	MS I	MS II	HPN IV
CHO	–	100	7.6	14.6	10.2
CHO	+	100	3.2	4.4	2.2
BHK	+	100	2.4	3.2	2.0

* Actm. is Actinomycin D.

As can be concluded from these data, MS I and HPN IV are present in approximately equal amounts, whereas MS II is slightly in excess. Actinomycin D seems to have no or only a small inhibitory effect on the synthesis of all substances. The apparent lower concentrations of these nucleotides in actinomycin treated cells compared to untreated cells is probably due to the shorter time of labeling (12 hr compared to 5 hr). There were no bacterial contaminations present in all cultures used in the above described experiments as tested by plating samples of 0.1 to 0.5 ml from the medium on a rich nutrient agar (NB, see Materials and methods). Likewise, no mycoplasma contamination could be detected by microbiological and biochemical methods (see Materials and methods). We therefore conclude that the unusual nucleotides found in mammalian cultures are synthesized by these cells. However, at this moment we cannot exclude the possibility that undetected viral infections are reponsible for their presence, even though there are no indications for this possibility.

In order to investigate whether or not the unusual nucleotides are present at all times or only formed at the end of logarithmic growth as it is in *B. subtilis* [1,2] the concentration of MS II and HPN IV as a function of cell number was measured in WI 38 cells. For that purpose, 12 Falcon flasks (25 cm^2 surface area) were inoculated with 1×10^3 cells/cm^2. To six of these flasks 1 mCi of H_3 [^{32}P] O_4 each was added. At different times, cells of one flask containing ^{32}P were used for extraction of low mol. wt substances and another flask without radioactivity was used for determination of cell numbers (see Materials and methods).

As can be seen in fig.2, WI 38 cells grow logarithmically for about 36 hr before they enter the stationary phase. During the following 84 hr one more doubling occurs. Following the synthesis of the unusual nucleotides, HPN IV and MS II, it can be seen (fig.2) that these nucleotides also increase linearly with time for 36 hr. The decrease from linearity occurs concomitantly with the end of logarithmic growth of WI 38 cells indicating, that both substances are synthesized in logarithmically growing and also in stationary phase cells. The GTP pool size, which was also measured in order to determine any influence of this nucleotide on MS II formation, shows the same kinetic as the unusual nucleotides. Its concentration is, however, more than 10 times higher. The ratio of the concentrations of MS II to GTP was at all times (between 0 and 120 hr) approx. 0.066 (from 0.059 to 0.071). A slightly higher

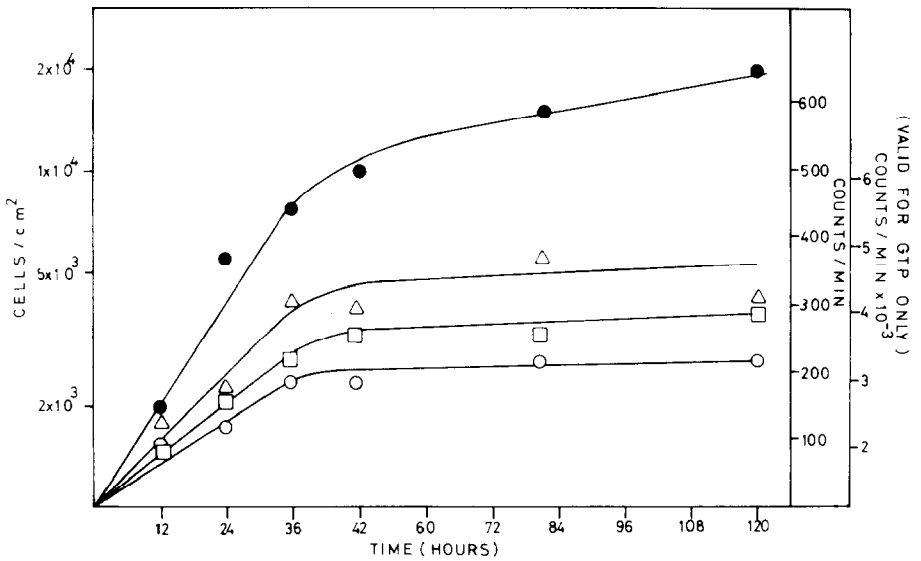

Fig.2. Growth of WI 38 cells in Eagles medium with additions (see Materials and methods) and pool sizes of GTP, HPN IV and MS II nucleotides. Growth (•—•) was determined with the aid of a Coulter Counter (△—△), HPN IV (□—□), and MS II (○—○) were extracted by formic acid and separated by thin-layer chromatography.

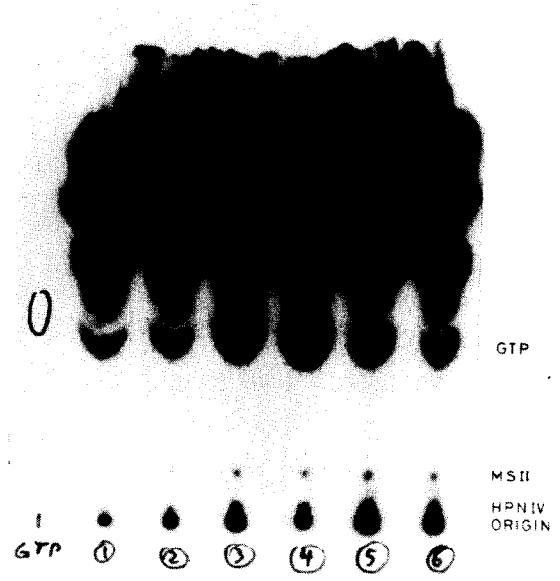

Fig.3 One-dimensional thin-layer chromatogram of formic acid extracts (10 μl) of WI 38 cells grown in the presence of H_3 [^{32}P]O_4. The chromatogram was exposed to Kodak non-screen X-ray film for 12 hr. The numbers represent samples taken at the same time as shown in fig.2. The increase in concentration of unusual nucleotides during increase in cell numbers is clearly visible.

ratio (0.070) was obtained for HPN IV and GTP.

These results show, that the concentrations of GTP, MS II and HPN IV are constant and approximately the same in each cell regardless of their status (dividing or not). It furthermore seems to confirm our results concerning the absence of bacterial contaminations.

In bacteria, the ratio of HPN to GTP increases drastically at the end of logarithmic growth, because of the drop in GTP pool sizes and the simultaneous increase in HPN [1,2].

Similar results were obtained with CHO and BHK cells. However, WI 38 cells accumulate ten times less MS I if any at all, than the other strains (see fig.3). The significance of this finding cannot be assessed at this moment.

Fig.3 shows the increase in HPN IV and MS II in logarithmically (column 1–3) and linearly (column 4–6) growing WI 38 cells. For quantitative determinations of HPN IV, chromatograms were allowed to develop longer (up to 3 hr) in 1.5 M phosphate, pH 3.4 in order to separate the spot at the origin and HPN IV more clearly. As in all previous experiments, no microbial contaminations or mycoplasmas were found by microbiological and biochemical methods (see Materials and methods).

4. Discussion

Our finding that the highly phosphorylated nucleotides (HPN) are involved in the control of development in *B. subtilis* [1–3], instigated us to investigate whether or not these nucleotides have similar functions in higher organisms. As has been described in this communication, these nucleotides are indeed present in mammalian cells in varying amounts. MS II and sometimes also MS I is also present in these organisms growing in cultures. This observation is important in so far as all three types of cells are from different organs of different organisms each showing high degrees of differentiation. Even though these cells can grow, they retain most of their characteristics as ovary (CHO), kidney (BHK), and lung (WI 38) cells. Compared to *B. subtilis* (if such a comparison is permitted at all), these cells are more similar to sporulating than to vegetative cells of *B. subtilis*.

Therefore it is not surprising that these nucleotides are found at all times in mammalian cells since they retain their organ specificity even when growing. Vegetative, that is growing cells of *B. subtilis*, differ completely from sporulating cells (no degree of differentiation).

If the HPNs are indeed involved in regulation of differentiation as it seems to be in *B. subtilis*, or in the maintenance of this state, one would expect to find these nucleotides at all times even in growing, but differentiated cells. This is indeed the case, at least in the investigated mammalian cells from as distinct organisms as hamsters and man.

At present we have not determined the different functions of the various nucleotides nor the reasons for their synthesis especially in these mammalian cells. We propose, in analogy to *B. subtilis*, that the HPNs somehow control differentiation, the MS nucleotides, in contrast, control other cellular functions, possibly RNA synthesis as proposed for *E. coli* [8].

Work is in progress in our laboratory investigating the above mentioned functions of regulatory nucleotides in mammalian cells.

The 27th generation of WI 38 cells (kindly provided by Prof. Hövel, Paul-Ehrlich Institut, Frankfurt/M., Germany) give exactly the same results as described above. The cells are also free of mycoplasmas as shown by immunofluorescence techniques and the uridine phosphorylase method (see Materials and methods).

Acknowledgement

This work was partially supported by the European Molecular Biology Organisation, EMBO. The author is grateful to Dr R. A. Lazzarini, Laboratory of Molecular Biology, National Institutes of Health, Bethesda, Md., for his permission to use his laboratory facilities for parts of this investigation. The very valuable suggestions made by Moss Jean Emeny (University of Leicester) for methods of detecting mycoplasma contaminations in tissue cultures are also gratefully acknowledged.

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