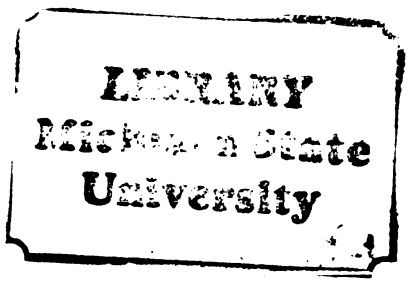

This is to certify that the

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STUDIES ON MESSENGER RNA METHYLATION

presented by

Sally Ann Camper

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of the requirements for

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Fritz M. Rottman
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STUDIES ON MESSENGER RNA METHYLATION

By

Sally Ann Camper

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ABSTRACT

STUDIES ON MESSENGER RNA METHYLATION

By

Sally Ann Camper

The major goal of my research was to understand the role of methylation in mRNA metabolism by focusing on the characterization of specific gene products. Initial characterization of two abundant bovine pituitary mRNAs was approached through analysis of their respective cDNA clones. A cDNA clone for prolactin mRNA was shown to be nearly full length by R-loop mapping. A growth hormone cDNA clone was subcloned into M13 and sequenced by the dideoxy chain terminator method. Determination of gene structure and sequence often provides information important for studying the mRNA products of the genes. A partial genomic clone for prolactin was obtained by screening a bovine genomic library, and portions of the prolactin gene were restriction mapped and subcloned. The primary sequence of the 5' flanking region, 5' untranslated region and a portion of the first intervening sequence was determined. Comparison of the bovine prolactin 5' flanking region with a published sequence for rat prolactin revealed several regions of potential regulatory significance.

Another phase of my studies involved the use of the methylation inhibitor S-tubercidinylhomocysteine to study the role of methylation in mRNA metabolism. S-tubercidinylhomocysteine was used to perturb mRNA methylation in primary pituitary cultures. Radioactive labeling studies indicated that cytoplasmic mRNA produced in the presence of S-tubercidinylhomocysteine was undermethylated at the 2'-O-methyl posi-

tion in the cap and deficient in N^6-methyladenosine. S-tubercidinylhomocysteine was also found to be extremely potent in HeLa cells for inhibition of N^6-methyladenosine methylation internally and at the cap site. The function of methylation in processing was probed by measuring the rate of cytoplasmic appearance of HeLa mRNA in the presence and absence of S-tubercidinylhomocysteine. The time of cytoplasmic appearance of undermethylated mRNA appeared to be delayed compared to normal mRNA. To test whether methylation affects mRNA stability, turnover of HeLa mRNA was measured. The half-life of the mRNA population was unaltered in cells treated with the inhibitor, compared to control cells. These studies on methylation of total mRNA have provided a framework for future examination of specific mRNAs in HeLa cells.

In memory of

Carolyn R. Camper

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LIST OF ABBREVIATIONS

STH	S-tubercidinylhomocysteine
m^6A	N^6-methyladenosine
HPLC	high performance liquid chromatography
tRNA	transfer RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
snRNA	small nuclear RNA
m^5U	5-methyluridine (ribothymidine)
m^1A	1-methyladenosine
m^2G	2-methylguanosine
Gm	2'-O-methylguanosine
Am	2'-O-methyladenosine
Um	2'-O-methyluridine
Cm	2'-O-methylcytidine
ψm	2'-O-methylpseudouridine
$m^2{}_2G$	N^2, N^2-dimethylguanosine
m^1G	1-methylguanosine
m^7G	7-methylguanosine
m^3C	3-methylcytosine
$m^6{}_2A$	N^6, N^6-dimethyladenosine
m^3U	3-methyluridine
m^5C	5-methylcytosine
NDV	Newcastle disease virus
VSV	Vesicular stomatitis virus

SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
ASV	Avian sarcoma virus
TRH	Thyrotropin releasing hormone
cDNA	complementary DNA
EDTA	Ethylenediamine tetraacetic acid
kb	kilobase
bp	base pairs
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid

BACKGROUND

FUNCTION OF POST TRANSCRIPTIONAL MODIFICATIONS OF RNA MOLECULES

Eukaryotic ribosomal, transfer, messenger and small nuclear RNAs all contain post-transcriptional modifications, the nature of which has been the subject of study for many years. Much still remains to be defined concerning the time sequence of these events and their role in the function and metabolism of specific RNA molecules.

I. Transfer RNA

Transfer RNAs are the most highly modified RNAs. The primary transcripts undergo 5' and 3' terminal trimming, and 3' terminal CCA addition. Numerous base and sugar modifications take place and some tRNAs undergo splicing to remove an intervening sequence. The methyl modifications include eight different base modifications and, in lesser abundance, 2'-O-methylation of ribose (Table 1). Overall there are approximately seven methylations per molecule. These post-transcriptional methylations have been proposed to be important for more efficient and accurate recognition of the tRNA. For instance, methylation could affect aminoacyl tRNA synthetase interactions, and wobble recognition might be amplified or restricted by modifications in the anticodon. Modifications may involve tRNA interaction with ribosomes including A and P site binding, translocation and termination (Kersten, 1982). In support of this idea it is interesting to note that two modifications, which arise from transglycosylation rather

Table 1

Distribution of methylated nucleosides in RNA

tRNA		rRNA 18S		rRNA 28S	
m^5C	17	Am	27	Am	30
m^2G	14	Um	25	Gm	25
m^5U	13	Gm	17	Cm	20
m^1A	12	Cm	17	Um	15
Gm	9	$m^6{}_2A$	5	m^1A	1.5
$m^2{}_2G$	8	m^6A	2.5	m^6A	1.5
m^1G	8	m^7G	2.5	m^5C	1.5
m^7G	7	Ψm	2.5	m^3U	1.5
Um	4			Ψm	1.5
m^3C	3				
Am	3				
Cm	2				

The percentage of the total (^3H)-methionine label that is found in each modified base or 2'-O-methylated ribose is tabulated above for mammalian tRNA and 18 and 28S rRNA (Munns and Liszewski, 1980).

than methylation (Q base and Y base), alter the efficiency with which charged tRNAs are utilized. Hypomodification of Y base results in increased usage whereas the opposite is true for Q base. It has been proposed that preferential utilization of hypomodified tRNAs could facilitate synthesis of specific proteins in tumor cells (Smith et al., 1983).

Microinjection of a gene for $tRNA^{tyr}$ into frog oocytes has facilitated analysis of the order and intracellular location of tRNA post-transcriptional events (Melton et al., 1980). Most of the base and sugar modifications occur in the nucleus and take place in a specific order correlating with other post-transcriptional processing steps such as the 5', 3' terminal trimming, 3' CCA addition and splicing (Figure 1). Since methylation events appear to take place in an obligatory order, the possibility exists that they facilitate recognition by enzymes responsible for other processing reactions.

Yeast mutants have been particularly useful in sorting out the function of some specific tRNA modifications and their relationship to other tRNA post-transcriptional processing events. m^5U, a modification that occurs prior to intervening sequence removal, appears not to be required for tRNA splicing or transport. In addition, these mutants appear to have no obvious physiological defect other than the absence of m^5U (Hopper et al., 1982).

Tissues undergoing rapid development have altered activity of tRNA methyltransferases and changes in tRNA methylation have been observed in malignant tumors (Kersten, 1982). The significance of these changes is not yet clear. A mammary adenocarcinoma deficient in

Figure 1

tRNA and rRNA processing pathways

The processing pathway for (A) $tRNA^{tyr}$ microinjected into frog oocytes is shown (Melton et al., 1981). The length of each transcript is given in nucleotides and base modifications are indicated as follows 5-methylcytosine (▫), pseudouridine (▽), m^1A (▼), and dihydrouridine (●).

The major rRNA pathways in (B) HeLa cells and (C) L-cells are shown with the sedimentation value for each intermediate (Lewin, 1980).

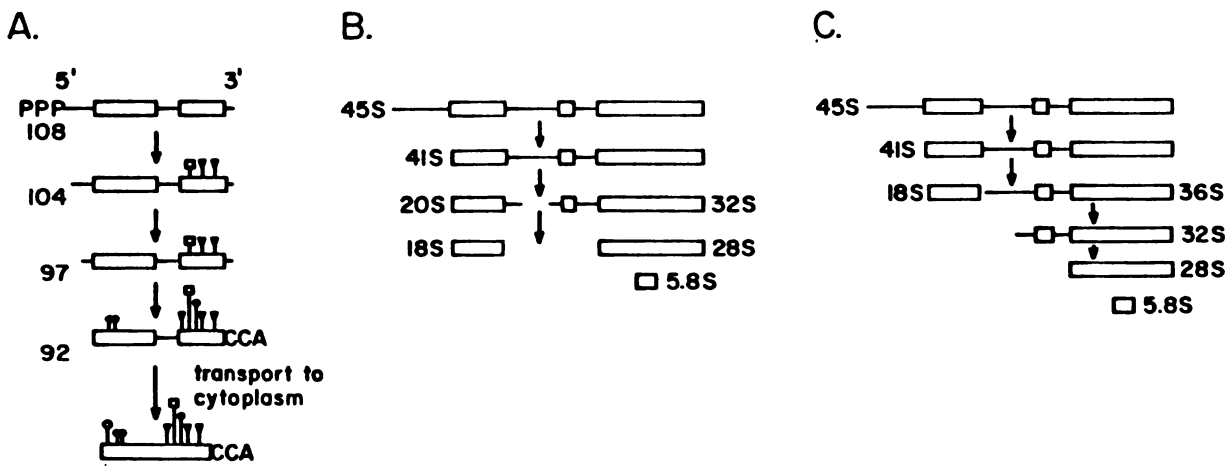
A.
5'
3'
PPP
108
104
97
92
CCA
transport to
cytoplasm
CCA
B.
45S
41S
20S
32S
18S
28S
5.8S
C.
45S
41S
18S
36S
32S
28S
5.8S

Figure 1

1-methyladenosine (m^1A) methyltransferase contains significant amounts of tRNA deficient in m^1A. The molecular weight of m^1A deficient tRNA is consistent with it being an unspliced precursor but it is not a substrate for tRNA processing enzymes. The mature cytoplasmic tRNA is somewhat deficient in m^1A also (Salas and Leboy, 1983). These observations imply that this particular base methylation may be important for efficient 5', 3' terminal processing or splicing of nuclear tRNA precursors, but not absolutely required.

II. Ribosomal RNA

Ribosomal RNA is transcribed as a 45S precursor containing 18S, 5.8S and 28S rRNA sequences separated by spacer regions as well as a 5' leader sequence. The processing pathway seems to be slightly different in different cells but generally to occur in an ordered manner (Lewin, 1980). The pathway for rRNA maturation in HeLa cells and L cells is illustrated in Figure 1. Ribosomal RNA is highly methylated, containing ribose and base methylations with the ribose methylations being the most abundant (Table 1). The 28S molecule contains 74 methylations per molecule and the 18S approximately 43 (Maden and Salim, 1974). The ribose methylations occur rapidly on nascent or newly synthesized transcripts, but the base methylations occur later in the maturation (Liau and Hurlbert, 1975). Although the methyl groups have been mapped to specific locations, they all do not occur within the same primary sequence (Maden and Salim, 1974), indicating either an incredible number of different methyltransferase enzymes or a type of recognition that does not involve primary sequence.

The function of rRNA methylation is still open to debate. 2'-O-methylation has been proposed to protect the rRNA from degradation because it inhibits cleavage by enzymes requiring cyclic phosphodiester intermediates. Base methylation may be important for tertiary structure necessary for ribosome function; this has been suggested for $m^6{}_2A$ located in a sequence near the 3' end of 18S rRNA (Gourse and Gerbi, 1980). Since it is clear that none of the spacer regions are methylated - all the methylation occuring on the 45S precursor are conserved in mature RNA molecules - it is possible that methylation is important for proper processing.

Experimental proof of any of these theories is fragmentary thus far. In experiments where HeLa cells were subjected to methionine starvation, 45S rRNA was synthesized but was approximately 80% deficient in methylation. The undermethylated rRNA could be cleaved to produce 32S, but no further, and the smaller cleavage product apparently was not stable (Vaughan et al., 1967). Similar results were obtained when ethionine, an inhibitor of methylation but not protein synthesis, was used in HeLa cells (Wolf and Schlessinger, 1977). These studies imply that methylation, although not essential for cleavage of 45S rRNA is important for cleavage of 32S rRNA and for stability of the RNA since the undermethylated species do not accumulate. However it is possible that perturbation of other aspects of metabolism by methionine starvation or treatment with ethionine are responsible for some of these effects on rRNA. For example, if a rapidly turning over protein were required for rRNA maturation, inhibition of protein synthesis by methionine starvation could block

processing. Also, a protein with ethionine incorporated instead of methionine could exhibit impaired function resulting in the apparent processing block. In this regard, a study involving a BHK cell line temperature sensitive for processing rRNA from 32S to 28S becomes particularly interesting. The rate of appearance of 28S rRNA in the cytoplasm is reduced 95% at the nonpermissive temperature and a 36S rRNA species is found, representing a shift to a less predominant pathway (Toniolo and Basilico, 1976). Because the methylation of 32S rRNA is unaltered at the nonpermissive temperature, it seems unlikely that the lesion is due to a methylation defect. The synthesis of small nuclear RNAs, which may be important in rRNA processing, also does not appear to be impaired. The defect appears to be in the activity of enzymes required for the synthesis of polyamines - ornithine decarboxylase and S-adenosylmethionine decarboxylase. These enzymes are subject to temperature sensitive synthesis or accelerated degradation (Levin and Clark, 1979). While this study does not rule out an important function for methylation in rRNA processing, it establishes the importance of polyamines in rRNA processing and underlines the dangers of utilizing agents to study RNA metabolism which also inhibit protein synthesis.

The methylation of rRNA in these ts BHK$^-$ cell mutants appears to be normal at the elevated temperature (Levin and Clark, 1979). Thus, 32S rRNA whose processing is blocked appears not to have an accelerated turnover in the case where it is methylated (Toniolo et al., 1973), but is wasted in a case where it is undermethylated (Wolf and Schlessinger, 1977). These observations correlate with a role for

methylation in nuclear stability of rRNA.

A role for methylation in nuclear events in rRNA metabolism, such as processing or stability does not rule out an additional role for methylation in rRNA function in the cytoplasm. Chick embryo fibroblasts treated with cycloleucine, an inhibitor of S-adenosylmethionine synthetase (Lombardini et al., 1973), have undermethylated rRNA in the cytoplasm which does not accumulate in ribosomes at the normal rate (Dimock and Stoltzfus, 1979). The nature of the undermethylation was not carefully examined, but did include a reduction in 2'-O-methyladenosine. Perhaps methylation of rRNA is necessary for production of functional ribosomes, although a direct inhibition of protein synthesis by cycloleucine can not be ruled out.

III. Small nuclear RNA

Small nuclear RNA (snRNA) is a class of RNA molecules found in the nucleus of eukaryotic cells, ranging in size from about 75 to 200 nucleotides in length, which is generally very abundant (10^4-10^6 copies per cell) and stable. The genes for snRNAs are apparently transcribed by RNA polymerase II which is also responsible for mRNA transcription, but they do not contain the "Hogness box" usually associated with genes coding for mRNAs and are not interrupted by intervening sequences (Roop et al., 1981). UV inactivation studies indicate that the transcription unit may be 5 kb (Elicieri, 1979), leaving open the possibility that snRNAs are cleavage products of larger RNA precursors. The transcription start site for U1 snRNA has been mapped to approximately 183 nucleotides upstream from the 5' end of the mature snRNA (Murphy et al., 1982). Therefore, the primary

transcripts must undergo a processing step to trim the 5' terminal extension.

Most snRNAs contain a methylated cap structure where 2,2,7-trimethylguanosine is coupled in a 5'-5' phosphodiester linkage at the first nucleotide of the chain. Small nuclear RNAs also contain all four 2'-O-methylated nucleosides positioned in the two nucleosides adjacent to the cap ($m^{2,2,7}{}_3$GpppNmpNmp...), similar to mRNA and internally, similar to rRNA and tRNA. Other post-transcriptional modifications include the base methylations m^6A and m^2G (Choi et al., 1982).

The function of snRNAs is still controversial. One snRNA (U_1) has been postulated to be involved in alignment of intervening sequences for cleavage of mRNA precursors (Murray and Holiday, 1979), possibly functioning as a coenzyme like the RNA factor in the tRNA processing enzyme RNAse P (Stark et al., 1978). It has been suggested that methylation could be a way of distinguishing between RNA strands for cleavage (Murray and Holiday, 1979). The existence of a snRNA identical to U1 but lacking the cap and first six nucleotides (Lerner et al., 1980) casts doubt on that theory. Another snRNA (U_3) may be involved in rRNA processing since it has been found in the nucleolus bound to 32S and 28S pre rRNA (Choi et al., 1982). Definitive evidence for involvement of these small nuclear RNA molecules in RNA processing is still not available. The function of post-transcriptional modifications of snRNAs is even more obscure.

IV. Messenger RNA

Messenger RNA, the least methylated of any of the RNA groups, is subject to several post-transcriptional processing events. Many mRNAs

undergo cleavage and splicing to remove intervening sequences as well as 3' terminal polyadenylation. The 5' end contains a cap - a 7-methylguanosine in a 5'-5' phosphodiester linkage with the first nucleotide of the mRNA. 2'-O-methylation occurs only on the nucleotides adjacent to the cap (e.g. m^7GpppN'(m)pN''(m)pNp...). Internally, the predominant methylation is m^6A, but in BHK cells m^5C can be detected (Dubin and Taylor, 1975). Knowledge of the relationship of methylation to other post-transcriptional processing events is crucial to understanding the regulation of gene expression, since splicing and polyadenylation have each been shown to be involved in regulating the level of expression of various genes (Nevins and Wilson, 1981; Darnell, 1982).

mRNA of higher eukaryotes is more methylated than that of lower eukaryotes. Cap structures that do not contain 2'-O-methylated ribose moieties (cap zero mRNA, m^7GpppNpNpNp...) are the predominant cap form in yeast and dictostelium, comprise a small fraction of caps in drosophila and are normally not found at all in animal cells (Lewin, 1980). Internal m^6A has not been detected in slime mold, yeast, and some plant mRNAs (Banerjee, 1980). The increase in methyl content of mRNA with an increase in complexity of the organism may argue for a regulatory role for mRNA methylation.

The order of post-transcriptional processing events for mRNA transcripts in the nucleus is beginning to be resolved. Capping is apparently a very early event in processing, occuring while the transcripts are still nascent chains (Babich et al., 1980). 2'-O-methylation of the N' position of the cap and m^6A addition are also nuclear events,

but 2'-O-methylation of the N'' position is catalyzed by a different cytoplasmic enzyme after incorporation of the mRNA into polysomes (Perry, 1981). The enzymology of the guanyltransferase and methyltransferases has been reviewed recently (Banerjee, 1980).

Some evidence has suggested that initiation of transcription could be dependent on addition of the m^7G cap structure (Jove and Manley, 1982, Chen-Kiang et al., 1982), but recent studies using an *in vitro* transcription system have shown that transcripts can be initiated and elongated to at least 20 nucleotides without the occurence of capping or 2'-O-methylation (Coppola et al., 1983). While transcription is apparently separable from capping and methylation, RNA polymerase II may exist in a complex with capping and methylation enzymes and thereby be stimulated in an allosteric fashion by SAM and its analogs (Furuichi, 1978; Wertheimer et al., 1980).

Polyadenylation probably occurs before splicing for a variety of HnRNA transcripts because examination of northern blots containing nuclear RNA that had been fractionated on oligo(dT)-cellulose reveals a large proportion of precursors in the poly(A) containing fraction (Schibler et al., 1978; Lai et al., 1978; Tilghman et al., 1978). Polyadenylation does not appear to be required for splicing, however (Zeevi et al., 1981). Evidence has begun to accumulate from *in vitro* transcription studies that implies that transcription proceeds beyond the polyadenylation site (Darnell, 1982). Perhaps poly(A) addition occurs following an endonucleolytic cleavage event which may not always be precise (Sasavage et al., 1982; Ahmed et al., 1982). In cases where mRNA undergoes differential splicing, selection of a

poly(A) site may then restrict the mRNA to a specific splicing pathway (Amara et al., 1982; Early et al., 1980). Transport to the cytoplasm apparently does not require polyadenylation, but non-adenylated RNA appears to be less stable (Darnell, 1982).

It is not known whether methylation is coupled in any way to polyadenylation. Transription of vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) in vitro results in large 3' terminal poly(A) (Rose et al., 1977; Weiss and Bratt, 1974). In VSV, large poly(A) was generated by in vitro transcription in the presence of S-adenosylhomocysteine. Cap methylation was also inhibited, suggesting a possible link between polyadenylation, ribose 2'-O-methylation and/or m^7G methylation. mRNA from Novikoff hepatoma cells and bovine pituitary cells deficient in ribose 2'-O-methylation did not exhibit an increased size when examined on sucrose gradients (Kaehler, 1973; S. Camper, unpublished results) indicating that the link between methylation and polyadenylation may be related to 5' m^7G methylation or may be peculiar to some viral systems.

Since the discovery that non-viral genes were interrupted by intervening sequences (Jeffreys and Flavell, 1977), it has been of interest to determine whether internal methylation could play a role in intervening sequence removal. Perhaps mRNA methylation works like a restriction-modification system in bacteria to designate regions to be cut or protected. Examination of intron-exon splice junctions has revealed a consensus sequence (Lerner et al., 1980) but homology with this sequence does not appear to be adequate for splicing.

N^6-methylation of adenosine results in a destabilization of A-U

base pairs (Engel and von Hippel, 1974, 1978) although they can still be formed. Thus, internal m^6A may be important in establishing the appropriate secondary structure for recognition by splicing enzymes. Precedence for an RNA processing enzyme being able to recognize secondary structure comes from the bacterial rRNA processing enzyme RNAse III (Robertson, 1982).

As mentioned previously, the role of methylation in mRNA splicing is still open to debate. Unlike the internal 2'-O-methylnucleosides of rRNA, internal m^6A methylation of mRNA does occur within a consensus sequence. Approximately 75% of the m^6A is found as G-m^6A-C and the remainder as A-m^6A-C in a diverse group of organisms including maize and HeLa cells (Nichols and Welder, 1981; Wei and Moss, 1977), indicating a conservation of sequence recognition. Many laboratories attempted to determine whether internal methylation was conserved during the maturation of mRNA (Lavi and Shatkin, 1975; Sommer et al., 1978; Chen-Kiang et al., 1979) in an analogous fashion to the conservation of internal methylation in ribosomal RNA maturation. The results were ambiguous, but recently it was clearly shown, for SV40 late RNA, that methylation occurs both within and outside of exons (Aloni et al., 1979). This result, however, does not preclude m^6A from involvement in recognition for splicing. In fact, some bacterial restriction enzymes recognize sequences distant from their cleavage sites (Roberts, 1980).

Internal methylation may serve a different function for different mRNAs. Viruses have many examples of differential processing and in some cases production of viral RNA requires that some RNA transcripts

remain unspliced. The location of m^6A, although not determined precisely, has been shown to roughly correlate with the location of splice junctions of some viral transcripts (Beemon and Keith, 1977; Canaani et al., 1979). Viral proteins may regulate the extent of splicing primary viral transcripts (Leis et al., 1980). There are cases of differential processing of non-viral transcripts including calcitonin (Amara et al., 1982), growth hormone (DeNoto et al., 1981), prolactin (Taylor et al., 1981), and immunoglobulin (Early et al., 1980), but it is not yet known how commonly this occurs or whether methylation is involved in the selection of the splice site.

It has been clearly demonstrated that capping and m^7G methylation are important features for efficient translation of mRNA on eukaryotic ribosomes (Both et al., 1975; Filipowicz, 1978; Kozak, 1978). Many viral systems have developed elaborate mechanisms to take advantage of the preference for translation of capped mRNA (Revel and Groner, 1978; Banerjee, 1980). 2'-O-methylation probably enhances binding of mRNA to ribosomes but its influence is less dramatic than m^7G methylation (Muthukrishnan, 1978).

Capping seems to be required for mRNA stability (Furuichi et al., 1977; Green et al., 1983). Perhaps other methylations such as cytoplasmic 2'-O-methylation and internal m^6A methylation play a role in cytoplasmic stability. There are several examples of situations where a specific mRNA appears to be preferentially stabilized or destabilized (Guyette et al., 1979; Heintz et al., 1983; Stiles et al., 1976; Bastos et al., 1977; Chung et al., 1981; Greenberg, 1972; Wiskocil, 1980). The mechanism by which this is accomplished remains

a mystery. Viral and host mRNA late in Herpes virus infection do not contain m^6A and late mRNAs have a shorter half-life (Bartkoski and Roizman, 1978). Alteration of methylation could be the cause of the different half-lives, but this hypothesis must be proven. There is no good evidence for an effect of internal methylation on translation. Heavily alkylated brome mosaic virus RNAs apparently are not prematurely terminated when translated in vitro (Fraenkel-Conrat and Singer, 1980). SV40 RNA is known to be hypermethylated in interferon treated cells and is not translated in cells under these conditions. However, the mechanism is unlikely to be via overmethylation of the SV40 messages because in vitro translation of the mRNA from interferon- treated SV40 infected cells did not appear different from control SV40 infected cells (Kahana et al., 1981). Although a function for internal m^6A in translation is not ruled out, it seems unlikely.

One approach to dissect the importance of methylation in RNA metabolism is to perturb methylation using an inhibitor and observe the consequences for mRNA metabolism. Several groups have utilized cycloleucine in this manner. Cycloleucine inhibits S-adenosylmethionine (SAM) synthesis (Lombardini et al., 1973) and should therefore be a general inhibitor of SAM dependent reactions. In rapidly growing cells SAM is partitioned 2:1 in favor of decarboxylation to produce polyamines rather than as a methyl donor for transmethylation reactions (Hoffman and Clark, 1983). Thus, reagents that deplete SAM necessarily impair polyamine biosynthesis. Polyamines have been shown to be important for rRNA synthesis (Levin and Clark, 1979), therefore

experiments involving the use of cycloleucine to inhibit methylation and study mRNA processing must be cautiously interpreted.

Bachellerie et al. (1978) showed that pulse-labeled heterogeneous nuclear RNA in cycloleucine treated cells was transiently of higher molecular weight than that of normal cells, and a role for methylation in efficient cleavage and splicing was suggested. Since a large portion of the HnRNA turns over in the nucleus (Salditt-Georgieff et al., 1981) and apparently normal RNA transcripts can be processed aberrantly (Dolan et al., 1983), the nature of the transient high molecular weight HnRNA requires further investigation. Perhaps cycloleucine is causing an increase in production of aberrant RNA transcripts which are then degraded in the nucleus.

Stoltzfus and Dane (1982), using cycloleucine, have shown that when avian sarcoma virus (ASV) RNA is 90% undermethylated, splicing of the viral transcripts is inhibited. These results imply a role for m^6A methylation in splicing.

We have used the methylation inhibitor S-tubercidinylhomocysteine (STH) to probe the function of mRNA methylation. STH is the 7-deaza analog of SAH and inhibits methylation competitively. It is stable in cells since it is not a substrate for SAH hydrolase (Chiag et al., 1977; Crooks et al., 1979). STH has been shown to inhibit methylation of tRNA (Crooks et al., unpublished results; Chang and Coward, 1975), catecholamines (Coward et al., 1974), dopamine (Michelot et al., 1977) and mRNA (Kaehler et al., 1977). STH does not cause significant inhibition of rRNA methylation (Rottman et al., 1979). DNA synthesis in lymphocytes is inhibited by STH (Chang and Coward, 1975).

In vitro studies have revealed a differential sensitivity of viral enzymes to STH. NDV and vaccinia virus (guanine-7-)methyltransferases were inhibited by STH with Ki of 0.4 μM and 140 μM, respectively (Pugh et al., 1977; Pugh and Borchardt, 1982). Vaccinia mRNA (nucleoside-2') methyltransferase was inhibited *in vitro* with a Ki of 1.2 μM (Pugh and Borchardt, 1982. Kaehler et.al (1979) found STH not to inhibit m^7G in Novikoff cells. This may be a reflection of the instability of the uncapped transcripts, or a difference in the Km's of the enzymes. The mammalian m^7G-methyltransferase is reported to be more like the vaccinia enzyme (Banerjee, 1980).

STH was shown to inhibit 2'-*O*-methylation and m^6A methylation in Novikoff cells (Kaehler et al., 1977). Undermethylated mRNA could be found in the cytoplasm on polysomes indicating that 2'-*O*-methylation and m^6A methylation were not strictly required for translation. Similarly, in cycloleucine treated cells, incorporation of mRNA into polysomes appeared normal (Dimock and Stoltzfus, 1979). These studies suggested that the selection of mRNA for translation is not influenced very much by 2'-*O*-methylation.

As polyamines have been implicated to be important in rRNA processing (Levin and Clark, 1979), it was crucial to determine whether STH perturbed polyamine biosynthesis in cells. Spermine and spermidine synthases, thought to be the rate limiting enzymes in polyamine biosynthesis, were both shown to be insensitive to inhibition by STH. In extracts of rat ventral prostate, 1 mM STH resulted in only 17 and 19% inhibition of these enzymes whereas SAH at the same

concentration inhibited by 7 and 47% respectively (Hibasami et al., 1980).

We have utilized STH to further explore the role of methylation in mRNA metabolism, using HeLa cells as a model system. We have also begun to develop a system for analysis of the post-transcriptional processing of two specific non-viral mRNAs, bovine pituitary prolactin and growth hormone.

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PART ONE

PARTIAL CHARACTERIZATION OF THE BOVINE PROLACTIN GENE

ABSTRACT

A bovine genomic library was screened with a nearly full-length prolactin cDNA clone. One positive prolactin genomic clone was restriction mapped, portions were subcloned into the plasmid vector pUC9, and exons partially mapped. The data indicate that a portion of the 3' end of the gene is missing and that the gene contains a minimum of 4 exons. A restriction fragment containing the 5' end of the gene was subcloned into the single stranded bacteriophage M13 and sequenced. The 5' flanking region of bovine prolactin was compared to the published sequence of rat prolactin. A region spanning 247 nucleotides flanking the bovine prolactin gene was approximately 87% homologous to the 5' flanking region of the rat gene, defining a region of potential regulatory significance.

INTRODUCTION

Post-transcriptional modifications of mRNA have been a primary interest of our laboratory and until about 1977, studies had largely been focused on bulk populations of mRNA. The advent of cDNA cloning technology made it possible to embark on the characterization of methylation and processing of a specific host cell mRNA. We chose the pituitary polypeptide hormone prolactin because it is a very abundant, regulated gene product. We selected bovine as a system to study due to the large size of the gland and to easy access to tissue from local slaughterhouses. A technique for maintaining monolayer cell cultures of bovine anterior pituitary glands was developed in our laboratory, and prolactin protein production in these cultures remains

high (Padmanabhan et al., 1982). A cDNA library was constructed from pituitary poly(A)$^+$ RNA by the dG-dC tailing technique. Prolactin cDNA clones were obtained (Nilson et al., 1980), and the largest cDNA clone, pBPRL72, was sequenced (Sasavage et al., 1982a).

We assumed that a very abundant protein would be well-represented in the mRNA population. In fact, cytoplasmic poly(A)$^+$ RNA from bovine pituitaries is very rich in prolactin mRNA (60-70%, Nilson et al., 1979). However, nuclear transcription studies in our laboratory indicated that the transcription rate of prolactin gene is low (0.04 to 0.065%, H. Meisner, unpublished results). Attempts to characterize the nuclear precursors for prolactin met with great difficulty, probably due in part to the low transcription rate of the gene. Other studies which required labeling the RNA in cell culture, such as the methylation analysis of a specific mRNA, were also technically not feasible. Meanwhile, techniques are being developed in our lab to analyze the methylation of a specific mRNA with <u>in vitro</u> labeling. We still are planning to characterize the post-transcriptinal processing of prolactin mRNA by reintroducing the prolactin gene into cells and thereby increasing the transcription of the gene.

We have undertaken the characterization of the bovine prolactin gene to provide information about the sequence and organization of the gene, to aid in interpretation of northern blot analysis of nuclear precursors, and to provide intervening sequence probes to rigorously define the processing steps.

Prolactin belongs to a family of evolutionarily related genes including pituitary growth hormone and placental lactogen (chorionic

somatomammotropin) (Niall et al., 1971). This fact, as well as the consideration that prolactin is regulated by many factors including cyclic AMP and ergocryptine (Maurer, 1981), glucocorticoids (Nakanishi, 1977), dopamine (Maurer, 1980), estrogens (Ryan et al., 1979), epidermal growth factor (Murdoch et al., 1982), calcium (White et al., 1981), and thyrotropin releasing hormone (TRH) (Potter et al., 1981; Biswas et al., 1982), make prolactin an interesting gene to study. Examination of primary sequence data from the 5' flanking portion the gene has revealed sites of potential regulatory significance.

MATERIALS AND METHODS

R-loop Analysis

The prolactin cDNA, pBPRL72 (1.25 μg), was linearized with EcoR1 and hybridized with 0.5 μg of pituitary polysomal poly(A)$^+$ RNA (Nilson et al., 1980) by a modification of a published procedure (Woolford and Rosbash, 1979). The hybridization was in a 50 μl reaction containing 70% formamide, 0.1 M Pipes pH 7.8, and 10 mM EDTA with the temperature decreasing in a step-wise fashion from 55^o to 45^{o}C over a 4 hour period. Then the samples were made 0.6% in glyoxal (Aldrich) and incubated one hour at 37^{o}C. The hybrids were diluted 0.6 fold, made 30 μg per ml in cytochrome c (Sigma) and spread on a hypophase of 10% formamide, 10 mM Pipes and 1 mM EDTA. The film of nucleic acid was picked up on parlodion coated copper grids, stained with uranyl-acetate, shadowed with platinum, and viewed in an electron microscope.

Genomic Blots

Pituitary genomic DNA (20 μg) prepared as described previously (Woychik et al., 1982) was digested with EcoR1 or BamH1 for 2 hours at 37°C with 5 units of enzyme per μg of DNA. Boiled pancreatic RNase (1 μg) was added to each reaction. Samples were electrophoresed in 0.7% agarose gels and blotted onto nitrocellulose (Millipore) or Gene Screen (New England Nuclear) using the method described by Southern (1975). Filters were baked 2 hours in vacuo, prehybridized for 4 hours at 42°C in 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 0.02 M sodium phosphate and salmon sperm DNA (100 μg per ml). Radioactive probe, prepared by nick translation of purified insert from pBPRL72, was added and allowed to hybridize 48 hours at 42°C. Filters were washed in 30 mM NaCl, 3 mM sodium citrate and 0.1% SDS at 65°C, and autoradiographed on Kodak XAR-5 film with Lightning-plus intensifying screens (DuPont) at -80°C for 3 days.

Genomic titration

Pituitary DNA (1-10 μg) and salmon sperm DNA (10 μg) (Sigma) containing 0 to 5 genomic equivalents of prolactin cDNA clone (pBPRL72) were digested with 5 units of EcoR1 per μg of DNA for 2 hours at 37°C. Digests were precipitated with ethanol, resuspended in 10 μl of 2 M NaCl, 0.1 M NaOH, boiled 2 minutes and spotted directly on a dry nitrocellulose filter (Millipore). The filter was washed briefly in 0.3 M NaCl, 0.03 M sodium citrate, baked 2 hour at 80°C in vacuo and prehybridized 4 hours at 42°C in the following buffer: 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 100 μg per ml salmon sperm DNA and 5x Denhardt's. The hybridization probe was made from

the insert of pBPRL72 by nick-translation (5.4×10^7 cpm per μg). Hybridization was for 48 hours at 42°C. Subsequently, the filter was washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1 % SDS at 68°C and autoradiographed. The radioactive spots were cut out and counted in a liquid scintillation counter.

RESULTS

R-loop analysis of prolactin cDNA

A prolactin cDNA plasmid (pBPRL72) was linearized by cleavage with EcoR1, hybridized with pituitary polysomal poly(A)$^+$ RNA and the hybrids visualized under the electron microscope. Digestion of pBPRL72 with Eco R1 results in a 5.4 kb linear DNA with 0.75 kb 5' and 3.6 kb 3' to the insert. Sequence analysis had previously established the 5', 3' orientation of the insert within the clone. A typical hybrid is illustrated in Figure 1. Many hybrids were examined, and although 3' extension of the RNA on the 3' side of the loop was observed, no extension on the 5' side was ever seen.

Prolactin gene number

The copy number of the prolactin gene was determined by a dot blot analysis (Figure 2). Varying amounts of pituitary DNA were spotted on a filter and as a standard salmon sperm DNA was spiked with varying amounts of the prolactin cDNA clone pBPRL72. For the purpose of constructing a standard curve, the bovine haploid genome size was taken to be 2.9×10^9 base pairs; thus 18.6 pg of pBPRL72 DNA added to 20 μg of salmon sperm DNA would yield one genomic equivalent. Three different amounts of bovine pituitary DNA were spotted on the filter to ensure that the response was linear. The filter was hybridized

Figure 1

R-loop of pBPRL72 with polysomal poly(A)$^+$ RNA

An electron micrograph of a typical hybrid between linearized prolactin cDNA (pBPRL72) and prolactin messenger RNA (Panel A) is interpreted schematically (Panel B). The cDNA is designated by solid lines and the messenger RNA, 5' to 3', is represented by a dotted line.

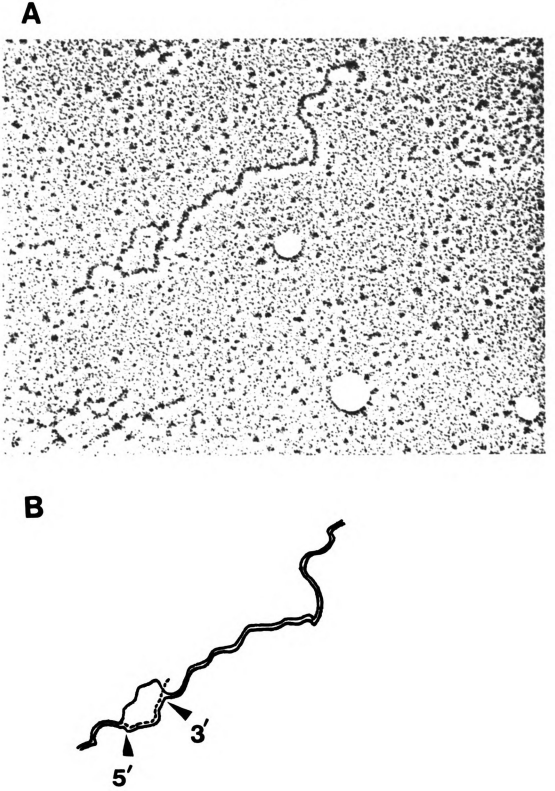


Figure 1

Figure 2

Genomic Titration

A dot-blot of 20 μg of salmon sperm DNA spiked with 0 to 5 prolactin genomic equivalents and 2, 5 and 10 μg of bovine pituitary DNA was probed with nick-translated PstI insert from the prolactin cDNA clone and autoradiographed. The standard curve was constructed by counting the spots from the dot blot in a liquid scintillation counter. The dashed line designated the hybridizable radioactivity in 10 μg of pituitary DNA.

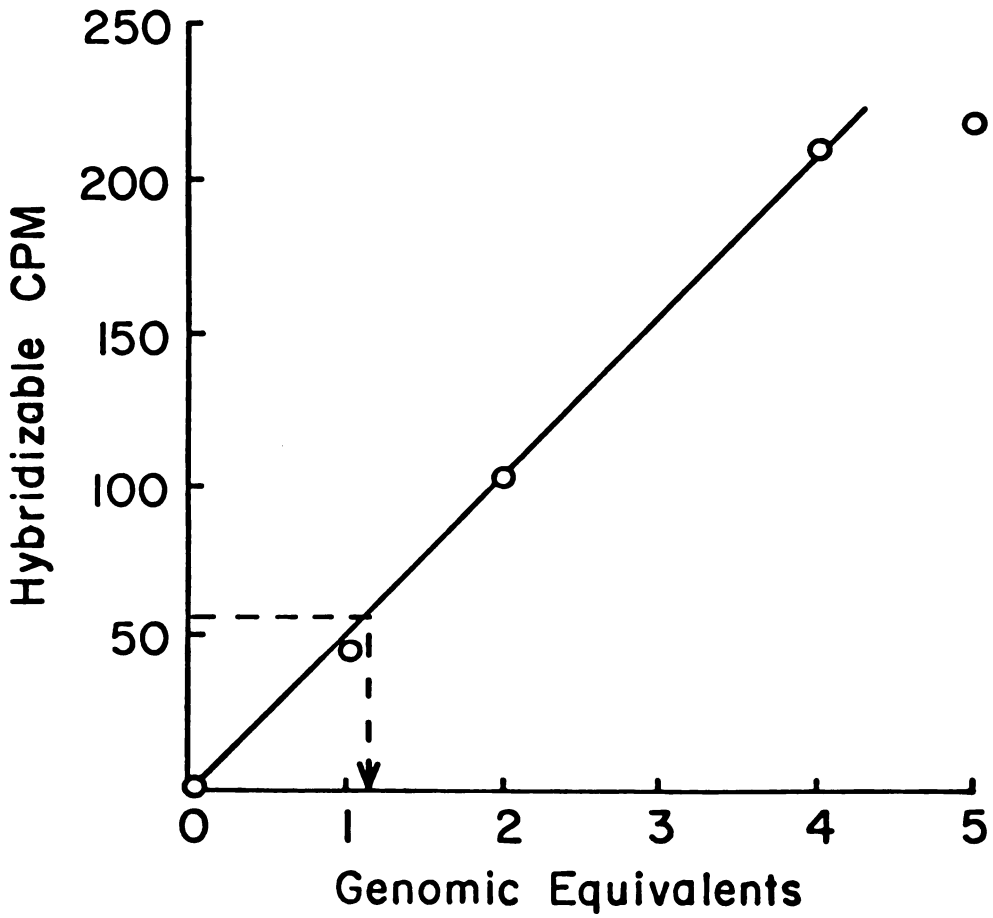

250
200
150
100
50
Hybridizable CPM
0
1
2
3
4
5
Genomic Equivalents

Figure 2

with nick-translated PstI insert from pBPRL72, washed, and autoradiographed. Then the radioactive spots were cut out and counted. The trace of background hybridization to 20 μg of salmon sperm DNA was subtracted and the standard curve constructed. The standard curve was linear except for the sample containing 5 genomic equivalents. Ten μg of pituitary DNA corresponded almost exactly to one genomic equivalent of prolactin and 5 μg corresponded to 0.5 genomic equivalents, indicating a single copy of the prolactin gene per haploid genome.

Genomic blots

The results obtained from Southern blots of restriction digests of pituitary DNA probed with the insert from pBPRL72 are summarized in Table 1. A digest with EcoR1 produced four distinct bands. Considering the fact that neither enzyme utilized cut within the cDNA, and that there is only one prolactin gene, it seems likely that there are a minumum of 4 exons, one on each of the hybridizing fragments. It is likely that there are 5 exons since the number of exons in a gene is usually conserved between species and the rat gene contains 5 exons (Chien and Thompson, 1980).

Isolation and characterization of a prolactin genomic clone

A bovine genomic library, constructed by insertion of placental DNA, partially digested with MboI, into the vector Charon 28 (Woychik et al., 1982), was screened with the insert from pBPRL72. Three prolactin positives were obtained. Restriction analysis with EcoR1 indicated that the prolactin positive clones were identical. One was selected for further analysis and designated λPro6.

Table 1

Genomic blots

Enzyme:	BamHI	EcoRI
MW: (kb)	15	
		7
		3
		1.5
		1

Southern blots of pituitary DNA digested with BamHI or EcoRI were probed with nick-translated insert from the full-length prolactin cDNA (pBPRL72). The genomic DNA fragments that hybridized were sized using a BamHI restriction digest of wild-type lambda for size markers. The sizes of the strongly hybridizing pituitary DNA bands are tabulated here in kilobase pairs (kb).

λPro6 was restriction mapped with single and multiple digests of HindIII, EcoR1, KpnI, BamHI, XorII, BglII and SmaI. To assist in ordering the fragments, Southern blots of restriction digests were probed with the insert from pBPRL72. The results of the restriction digests and Southern blots are indicated in Table 2. Restriction fragments greater than 13 kb or less than 2 kb were inaccurately sized on some gels. This data contributed to construction of the map of λPro6 illustrated in Figure 3. The 0.64 kb EcoRI fragment could not be placed unambiguously but is likely to lie towards the 3' end of the bovine DNA.

Briefly, the logic for constructing the map was as follows: digests with HindIII, KpnI, and EcoRI set the distance of the first HindIII and EcoRI sites from the known KpnI site in the left arm of the vector. Double digests and Southern blots with HindIII and EcoRI oriented those restriction sites within the bovine DNA. The locations of the BglII sites were tenatively placed based on knowledge of the location of BglII sites within the Charon 28 vector (Rimm et al., 1980), and based on a BglII HindIII double digest which contained some partial digestion products (data not shown).

To obtain the orientation of the prolactin gene within the λPro6 clone, HindIII and EcoR1 digests were probed with the intact insert of pBPRL72 or small fragments of the insert purified by standard techniques (Maniatis et al., 1982). The various probes used are illustrated in Figure 4 with the restriction map of pBPRL72. As a control for fragment purity, restriction digests of the pBPRL72 clone were included in the blots. The results, summarized in Table 3, estab-

Table 2

Restriction digests and Southern blots of λPro6

HindIII	EcoRI HindIII	EcoRI	KpnI	KpnI HindIII	KpnI EcoRI	BamHI	KpnI BamHI	BglII	SmaI	XorII (PvuI)
20	20	20	20	17.6	17.0	20	20	23	20	23
14				13.5*					13*	
	11*	7.6*					8			11.1
					6.8			6.7		
6				5.9	6.0*					
				5.6					5.7	
4.4*	4.4*			4.4*						
								3.3*		
		3.0								
	2.9									
	2.6				2.7			2.05*		
	1.4*	1.4*			1.5*			1.0		
	0.64	0.64						0.6		
	0.48									

λPro6 was digested with the indicated restriction enzymes and run on agarose gels containing ethidium bromide. The bands were visualized with ultraviolet light and the sizes estimated by comparing their migrations to those of molecular weight markers. The estimated sizes in kilobases are tabulated above. The agarose gels were blotted onto nitrocellulose by the Southern technique, and probed with the insert from pBPRL72. Bands which were hybridizable to the probe are indicated with an asterisk (*).

Figure 3

λPro6 Restriction Map

The prolactin cDNA clone (pBPRL72) is shown oriented in the 5' to 3' direction with potential intervening sequence borders marked. A and B refer to the small fragments used as probes to orient the gene (Table 3). Below is shown a preliminary restriction map of λPro6 with the following sites and their abbreviations: KpnI (K1), SmaI (S1), HindIII (H3), EcoRI (R1), BamHI (B1), BglII (B2), and XorII (X2). The bovine DNA is depicted as a single line, and the vector Charon 28 as a double line. The locations of four exons have been tentatively assigned and are indicated with dark boxes. Under the map for λPro6 are the HindIII subclones in pUC9 pλP2, pλP6 and pλP12. The pλP12 subclone has been additionally mapped for PstI (P1) and PvuII (P2).

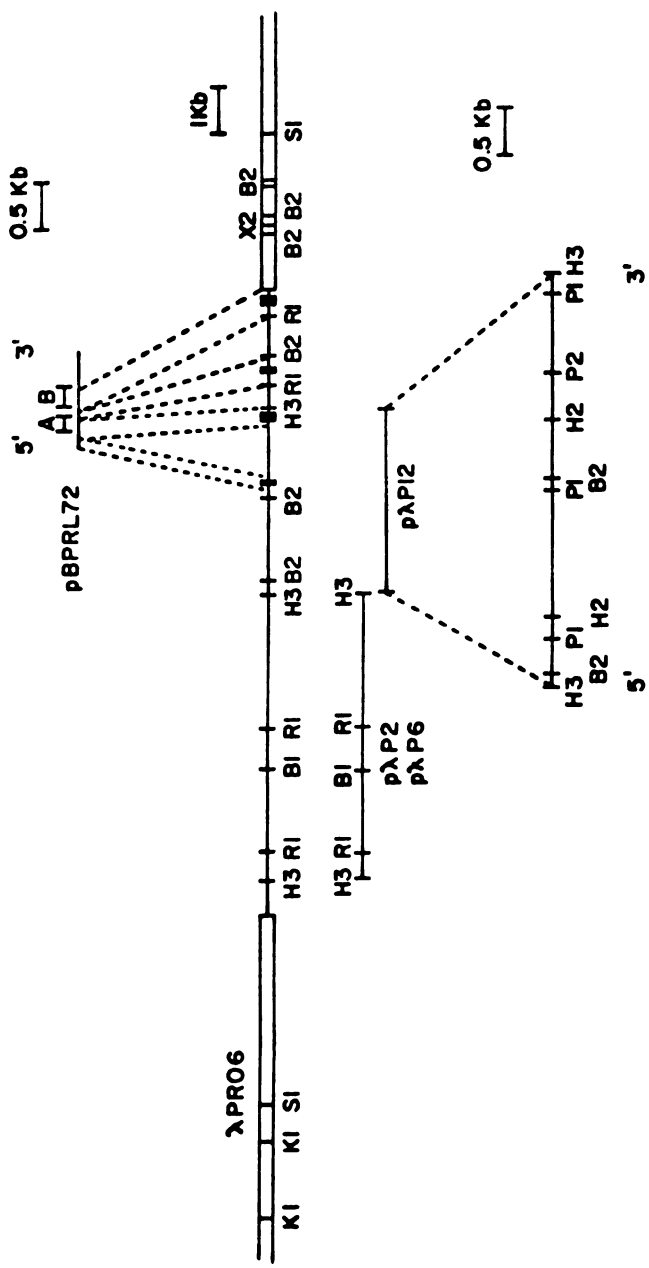

0.5 Kb
1Kb
0.5 Kb
5'
3'
A
B
pBPRL72
λPRO6
K1
K1
S1
H3
RI
H3
RI
BI
RI
H3
B2
B2
X2
B2
B2
S1
H3
RI
B2
RI
pλP2
pλP6
H3
RI
BI
RI
pλP12
H3
B2
PI
H2
P2
PI
H3
3'
H3
PI
B2
H2
5'

Figure 3

Figure 4

Restriction map of pBPRL72

Restriction sites predicted from the published sequence (Sasavage et al., 1982a). Fragments purified and used as probes are indicated below the restriction map and the coordinates of each fragment are indicated in parentheses. Potential exon junctions predicted by comparison to the rat prolactin gene (Cooke and Baxter, 1982) are indicated with hatched lines.

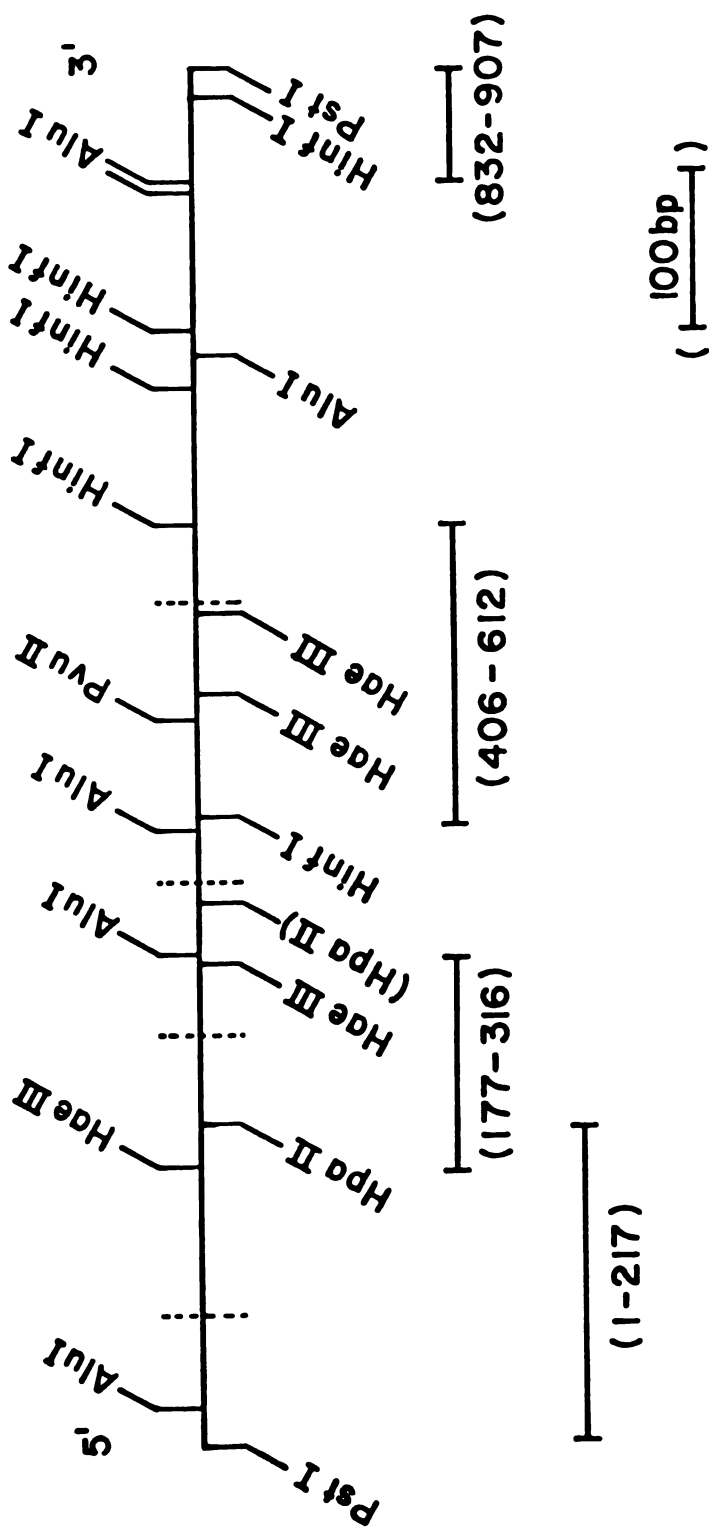

5'
Pst I
Alu I
Hpa II
Hae III
Alu I
Hae III
Alu I
(Hpa II)
Hae III
Alu I
Hae III
Pvu II
Hae III
Alu I
Hinf I
Alu I
Hinf I
Hinf I
Hinf I
3'
Pst I
Hinf I
Alu I
(1-217)
(177-316)
(406-612)
(832-907)
100bp

Figure 4

Table 3

Mapping exons in λPro6

PROBE:	A (177-316)		B (406-612)			C (1-907)		
RESTRICTION ENZYME:	H3	R1	H3	H3/R1	R1	H3	H3/R1	R1
FRAGMENT SIZES (kb)	20	20	20	20	20	20	20	20
	*14	11	*14	*11	*12	*14	*11	*12
		*7			7.6			*7.6
	6.1		6			6		
	*4.5		4.4	4.4		*4.4	*4.4	
		2.7			3.0			3.0
				2.9			2.9	
				2.6			2.6	
		*1.4		1.4	1.4		*1.4	*1.4
				0.64	0.64		0.64	0.64
				0.48			0.48	

λPro6 DNA was digested with the indicated restriction enzymes- HindIII (H3), EcoRI (R1) - and run on agarose gels containing ethidium bromide. The sizes of the fragments that were visualized under ultraviolet light are tabulated above. These restriction digests were transferred to nitrocellulose and analyzed by Southern blotting. Replicate blots were probed with different radiolabeled fragments from pBPRL72; nt 177-316 (A), nt 406-712 (B), or the entire pBPRL72 insert, nt 1-907 (C). Bands containing DNA hybridizable to the probe are denoted with an asterisk (*).

lished the orientation of the gene within the clone. The 5'-most probe (A, in Figure 3) spanned at least 2 exons, hybridizing to a 4.5 kb HindIII fragment, and to the right arm of Charon 28, as well as to a 7 and a 1.4 kb EcoRI fragment. A probe from the mid-portion of the cDNA (B, in Figure 3) was predicted to span 2 exons, but only hybridized to a single band containing the right arm of Charon 28. This information established the orientation as 5' to 3' left to right in the Charon 28 vector (Figure 3).

An identical blot containing HindIII and EcoR1 digests of λPro6, and a double digest of pBPRL72 with PvuII and PstI as a control, was probed with an AluI/PstI fragment corresponding to 74 nucleotides at the 3' end of of the cDNA insert (Figure 4). This probe did hybridize to the control 632 bp PvuII/PstI fragment of pBPRL72, but it failed to hybridize to any of the bands from λPro6 indicating that the 3'-most region of the cDNA was not represented in the λPro6 genomic clone.

To facilitate restriction mapping, a HindIII digest of λPro6 was subcloned into the plasmid vector pUC9 (Vieira and Messing, 1982). The assignment of EcoRI sites and the BamHI site of λPro6 was confirmed by cutting two clones containing the 6 kb HindIII fragment in opposite orientations (pλP2, pλP6) with EcoRI and BamHI (Figure 3). Since this region represents 5'-flanking DNA, it was not mapped further. A more detailed restriction map for the 4 kb HindIII fragment was constructed using the pλP12 subclone (Figure 3). In addition to single digests, double digests with HindIII, PvuII, EcoRI, BglII, HincII and PstI were performed in several combinations to confirm the position of the restriction sites. Ordering of the fragments in pλP12 was facilitated

by Southern blot analysis of some of the restriction digests (data not shown) which were probed with a 5' PstI/HpaII fragment of pBPRL72 spanning nucleotides 1-217 (Figure 4). The location of the 5'-most exon was deduced to fall within an approximately 0.8 kb BglII/HincII site in pλP12.

Primary sequence of the 5' flanking region

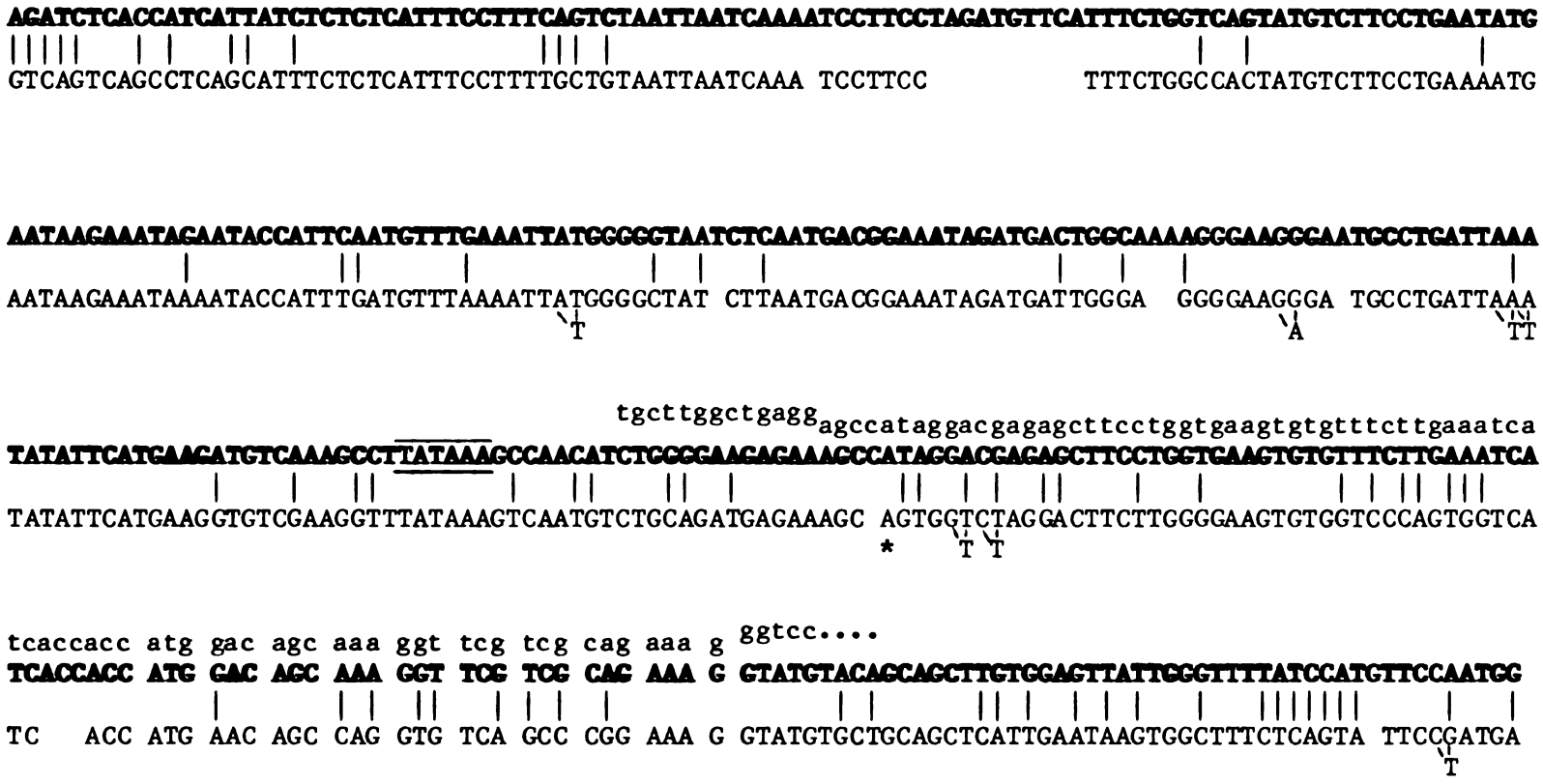
The 5' most exon was mapped to within the approximately 800 bp BglII HincII fragment in the subclone pλP12. The 2 kb HincII fragment containing this section was gel purified, cut with BglII, and the resulting fragments subcloned into the BamHI-SmaI site of the M13 bacteriophage vectors mp8 and mp9 (Messing and Vieira, 1982). Two clones positive for pBPRL72 sequence were selected for sequence analysis. The orientation of the single-stranded clones is such that they are complementary. To date 400 base pairs of each clone has been sequenced by the dideoxy method (Sanger et al., 1977), but no overlap has yet been obtained.

The preliminary sequence information generated has revealed that further digestion of the 2 kb HincII fragment with AluI should produce several smaller clones whose sequence would provide overlap and the necessary confirmation of the sequence from the opposite strand. The sequence of the 5' flanking region, first exon and intervening sequence junction was obtained from examination of multiple gels beginning at the BglII-BamHI junction and extending various distances downstream (rightward, Figure 3). The sequence of the messenger sense strand is shown in Figure 5, and is compared to the 5' portion of the prolactin cDNA (pBPRL72). Approximately 13 nucleotides at the 5' end

Sequence of the 5' portion of the bovine prolactin gene and comparison with rat prolactin

A BglII-HincII restriction fragment from pλP12 (approximately 0.8 kb) was subcloned into the bacteriophage M13mp8 to provide a single stranded template. The sequence of the messenger sense of a portion of the bovine prolactin gene was determined by dideoxy sequencing and is shown in the figure (bold face). The region homologous to the canonical TATAAA sequence is underlined. The sequence of the bovine prolactin cDNA pBPRL72 (Sasavage, 1982a) is in lower case letters, juxtaposed with an homologous sequence in the gene. The points of divergence have been emphasized by elevating the lower case cDNA sequence.

A portion of the rat prolactin gene (Cooke and Baxter, 1982) is given in upper case letters below the bovine sequence for purposes of comparison. The position of the rat prolactin cap site is indicated with an asterisk (*). The rat prolactin sequence has been aligned to allow maximum homology; points of mismatch are indicated by vertical lines.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AATAAGAAATAGAATACCATTCAATGTTTGAAATTATGGGGGTAATCTCAATGACGGAAATAGATGACTGGCAAAAGGGAAGGGAATGCCTGATTAAA
AATAAGAAATAAAATACCATTTGATGTTTAAAATTATGGGGCTAT CTTAATGACGGAAATAGATGATTGGGA GGGGAAGGGA TGCCTGATTAAA
T
A
TT
tgcttggctgagg
agccataggacgagagcttcctggtgaagtgtgtttcttgaaatca
TATATTCATGAAGATGTCAAAGCCTTATAAAGCCAACATCTGCGGAAGAGAAAGCCATAGGACGAGAGCTTCCTGGTGAAGTGTGTTTCTTGAAATCA
TATATTCATGAAGGTGTCGAAGGTTTATAAAGTCAATGTCTGCAGATGAGAAAGC AGTGGTCTAGGACTTCTTGGGGAAGTGTGGTCCCAGTGGTCA
*
T
T
tcaccacc atg gac agc aaa ggt tcg tcg cag aaa g
ggtcc....
TCACCACC ATG GAC AGC AAA GGT TCG TCG CAG AAA G GTATGTACAGCAGCTTGTGGAGTTATTGGGTTTTATCCATGTTCCAATGG
TC ACC ATG AAC AGC CAG GTG TCA GCC CGG AAA G GTATGTGCTGCAGCTCATTGAATAAGTGGCTTTCTCAGTA TTCCGATGA
T

Figure 5

of the cDNA do not correspond to any of the genomic sequence obtained thus far. The location of a potential TATAAA sequence is underlined. This sequence is apparently part of the eukaryotic promotor (Corden et al., 1980). The CAAT homology unit (Benoist et al., 1980), which may serve to enhance transcription, appears to be lacking in the 5' flanking region of the bovine prolactin gene.

DISCUSSION

Full length prolactin cDNA

A cDNA clone for prolactin was isolated and sequenced (Sasavage et al., 1982a). This clone is thought to be nearly full-length by several criteria. First, the size of the insert is 907 base pairs which corresponds well with the estimate of a mRNA of approximately 1000 nucleotides, assuming the poly(A) tail is 100 to 150 nucleotides. The clone contains a small portion of the poly(A) tail, thus any sequence not contained within the clone must be from the 5' end of the mRNA. Secondly, the 5' untranslated region of bovine prolactin included in pBPRL72 is 67 nucleotides long. The rat prolactin mRNA has only 51-52 nucleotides in its 5' untranslated region (Maurer et al., 1981). In the case of pituitary growth hormone and alpha subunit to the glycoprotein hormones, the length of the 5' untranslated region is conserved between species differing by only 1-2 nucleotides (Fiddes and Goodman, 1981; DeNoto et al., 1981). It appears that the bovine prolactin mRNA has at least 15 more nucleotides in the 5' untranslated region than the rat prolactin mRNA sequence. Length is apparently not conserved for bovine and rat prolactin, but it seems likely that

pBPRL72 is very close to being full-length. Finally, electron microscopic analysis of hybrids formed between pBPRL72 DNA and pituitary mRNA did not reveal an unhybridized 5' terminal extension, indicating that any additional sequence not contained in the cDNA clone is probably less than 50 nucleotides (Figure 1). The knowledge that the cDNA clone was nearly full-length facilitates analysis of the gene organization.

Prolactin gene number

Prolactin mRNA has been shown to exist as several polymorphic variants (Sasavage et al., 1982a) and to contain multiple poly(A) sites (Sasavage et al., 1982b). Although multiple poly(A) sites could be observed in mRNA from a single individual, the possibility still exists that the variation in the poly(A) site resulted from differences in nonallelic or duplicate loci for the prolactin gene rather that from a processing event. Considering the evidence for polymorphism and multiple poly(A) sites, we felt that it was necessary to definitively show that there was a single bovine prolactin gene. The rat prolactin gene has been assigned as a single-copy gene (Cooke and Baxter, 1982), but that was based on genomic blotting data which could not rule out the possibility of multiple prolactin genes embedded within similar surrounding DNA. To clarify this point for the prolactin gene, we titrated the gene number using a dot blot procedure (Figure 2). The data indicates that there is a single copy of the bovine prolactin gene per haploid genome. There is a potential for prolactin-like genes, pseudogenes or homologous genes, but these are not detected at the stringency of hybridization we used.

Genomic organization

A bovine genomic library screened for prolactin produced three positive clones which appeared identical by restriction digestion. One positive, designated λPro6, was restriction mapped and partially sequenced. The restriction map was confirmed in part by mapping the HindIII subclones (pλP2, pλP6, pλP12). The portion of the map extending from the 3'-most HindIII site to the junction with Charon 28 is not precise due to the inaccuracy in sizing large restriction fragments containing the right arm. We are planning to confirm the map of λPro6 in this region by subcloning BglII, SmaI, and EcoRI digests of λPro6 into the vector pUC9 and restriction mapping them. The placement of the second exon (Figure 3) is still ambiguous. It is possible that the 4 kb HindIII fragment contains a single exon. The location of the exons are being confirmed by sequence analysis and additional Southern blots.

The 3' portion of the prolactin gene appeared to be missing from λPro6 based on Southern blots probed with a fragment corresponding to the 3' end of the cDNA. It is possible that one of the prolactin positive genomic clones contained additional 3' sequence, hybridizable to the 3'-most portion of pBPRL72, that was overlooked in screening the clones only by restriction mapping. The prolactin positive clones were analyzed by a dot-blot test and probed with the same 3' probe (nucleotides 832-907, Figure 4). Clearly, none of the prolactin clones contained additional 3' sequence (data not shown).

At least 4×10^5 plaques were examined in the screening and, for an insert size of 15 kb, the probability of finding a single copy gene is

86%. We repeated the screening, and did not find any clones extending into the 3' flanking regions. Additionally, we screened the unamplified library, in case clones containing this portion of the gene were poorly amplified and then lost; however, we did not obtain additional clones. There are several possible explanations for the apparent underrepresentation of a sequence in a library. First, a portion of the sequence may be deleterious to the growth of lambda, and thus not produce a viable clone. Secondly, there may be homology between two regions of a clone, resulting in its being lost through recombination. Finally, there may be a wealth of MboI restriction sites in the 3' terminal region giving small fragments with a low probability of cloning. We are attempting to locate a clone containing the missing portion of the prolactin gene by screening bovine genomic libraries constructed with other restriction digestions.

Since the prolactin cDNA exhibited extensive polymorphism, and the DNA for the genomic blots did not come from the same individual as that used for the cloning, the λPro6 map will be confirmed by examining genomic blots of DNA isolated from semen samples of a variety of cattle.

Sequence of the 5' portion of the bovine prolactin gene

The sequence of the 5' flanking and the 5' untranslated regions and a portion of the signal peptide of the bovine prolactin gene has been determined by sequencing a portion of a BglII-HincII fragment cloned into M13mp8. The sequence of the sense strand is shown in Figure 5. Alignment with the cDNA sequence reveals the presence of a 13 nucleotide extension of the 5' terminus of the cDNA. These 13

nucleotides could represent another exon, but it seems more likely that the extension is a result of a cDNA cloning artifact. Synthesis of the first strand from the mRNA template was primed by oligo(dT) and extended close to the cap site. Then, following alkaline hydrolysis of the mRNA template, the second strand was synthesized utilizing a "hook" at the 5' end as a primer for DNA polymerase I. Perhaps generation of the 5' hook results from slippage of the reverse transcriptase and misincorporation at the 5' end. Then limited S_1 digestion to open the hook could leave unpaired ends which could be repaired by DNA polymerase I (Richards et al., 1979). Experiments in progress to map the location of the cap site by primer extension will clarify this point.

Comparison of the BamHI and EcoRI genomic blots (Table 1) with the restriction map of λPro6 allows us to predict the gene size. The position, but not the length, of intervening sequences is usually conserved, therefore we may find the bovine gene to be larger or smaller than the 10.5 kb rat prolactin gene (Cooke and Baxter, 1982). Since the BamHI digest produced only a single band of approximately 15 kb, we could estimate the gene size to be less than 15 kb. The orientation of the 3 EcoRI bands from the genomic blot is 5' to 3' are 7kb, 1.5 kb, and then either the 1.0 or 3.0 kb, based on λPro6 map (Figure 3). Following the 1.5 kb EcoRI fragment could be the small 0.64 kb EcoRI piece that was not placed. The location of the BamHI site is less than 1 kb to the 5' side of the 7 kb EcoRI fragment. Assuming that the 5' end of the gene corresponds to the section of the BglII-HincII fragment that was sequenced, the 5' end of the gene is 6 kb

into the BamHI fragment. The EcoRI fragment and the 3' most exon could be arranged to yield a minimum gene size of 6 kb. The maximum size would be approximately 9 kb.

Comparison of the bovine prolactin 5' flanking region with other genes

Sequences that are functionally important may evolve less rapidly than those which are less important (Wilson et al., 1977). Comparison of DNA sequences from related genes or from the same gene in different species can reveal conserved regions of potential regulatory significance (Woychik et al., 1982; Nakanishi et al., 1981). However, this is not always true (Heilig et al., 1982). We compared the 5' flanking region of the bovine prolactin gene with a sequence published for rat prolactin (Cooke and Baxter, 1982) (Figure 5). Surprisingly, there was extensive homology between the two species starting at the cap site for rat prolactin and extending upstream for about 247 nucleotides, whereupon the sequences appear to begin to diverge. By aligning the sequences to obtain maximal overlap, the homology in the 5' flanking region is greater than 87%. This is remarkable, considering that there is only 68% homology between the exon regions of rat and bovine prolactin (Sasavage et al., 1982a).

Cooke and Baxter (1982) proposed that the 5' end of the rat prolactin gene may have had an independent origin. This assertion was based on the presence of a 10 base pair repeat (GAAGAGGATG) located in the 5' flanking DNA 77-68 base pairs upstream from the cap site and at bases 340-349 in the first intervening sequence. Since the 5' flanking regions of rat and bovine prolactin are very homologous on each side of the 10 base pair sequence, then insertion of the first

exon, if it occured, must have taken place prior to the divergence of cattle and rats.

We compared the 5' flanking region of the bovine prolactin gene to the published sequence for the bovine growth hormone gene (Woychik et al., 1982). A 38 bp sequence located 100 bp upstream from the cap site in the bovine growth hormone gene may be involved in regulation of the growth hormone gene. No significant homology to the 38 bp sequence was detected in the bovine prolactin gene. This may not be surprising since prolactin is negatively regulated by glucocorticoids and growth hormone is positively regulated. However, the sequence reported to be important in positive regulation of ovalbumin by progesterone (Compton et al., 1983) has been found in two copies oriented head-to-head near the casein genes, which are negatively regulated by progesterone (Jones et al., 1983).

Identification of regions of DNA that are required for hormonal regulation can be approached in several ways. While sequence comparisons can define regions of potential significance, in vitro mutagenesis, gene transfer (Robins et al., 1982) and hormone receptor binding studies (Payvar et al., 1981) must be done to prove the involvement of a DNA sequence in gene regulation. Hormone receptor binding studies for progesterone have produced conflicting results (Compton et al., 1983; Mulvihill et al., 1982), which can probably be resolved using in vitro mutagenesis and gene transfer. The striking degree of homology between the rat and bovine prolactin 5' flanking regions defines an area of potential regulatory significance which can now be tested.

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PART TWO

SEQUENCE OF BOVINE GROWTH HORMONE mRNA

ABSTRACT

A nearly full length cDNA clone for bovine growth hormone mRNA has been subcloned into the bacteriophage M13 and sequenced by the dideoxy chain terminator technique. The cDNA clone codes for a leucine at amino acid 121. Comparison of the sequence of our growth hormone cDNA clone with a previously published sequence revealed a possible nucleotide polymorphism in the codon for serine 55.

INTRODUCTION

To further study post-transcriptional modifications of mRNA, it became necessary to focus on the processing of specific cellular gene products. We chose to work on the pituitary gene product growth hormone because it is an abundant mRNA in the adult pituitary representing approximately 25% of the poly(A)$^+$ RNA (Nilson et al., 1983), and it is subject to regulation by glucocorticoids, thyroid hormone (Spindler et al., 1982) and other factors. Many of the studies we planned would be facilitated by having cDNA clones for the mRNA. These clones could be used to enrich for specific mRNA sequences, to rapidly make very pure radioactively labeled probes, and as a basis for mapping the location of internal m^6A within the mRNA.

We constructed a cDNA library from bovine anterior pituitary mRNA by cloning double-stranded cDNA into the PstI site of the plasmid pBR322 (Nilson et al., 1980b). The library was screened with a ^{32}P-cDNA probe made from partially purified growth hormone (Nilson et al., 1980a). A growth hormone clone was obtained, and the identity verified by positive selection and cell-free translation. Sequencing was begun

on the clone, but before it could be completed, the entire sequence of another bovine growth hormone cDNA was published by another group (Miller et al., 1980). Sequencing efforts were abandoned at that point.

In the course of other experiments, it became necessary to have a higher specific activity growth hormone probe. This was achieved by subcloning the GH cDNA, designated pG23, into the bacteriophage M13 to produce single-stranded templates for synthesis of radioactive probes (Messing and Vieira, 1982). I decided to verify the identity of the subclones by rapid dideoxy sequence analysis (Sanger et al., 1977). Surprisingly, three of the first 20 nucleotides differed from the published growth hormone sequence. This finding, coupled with the knowledge that the prolactin gene contained an unexpected amount of polymorphism (Sasavage et al., 1982a), led me to sequence the entire bovine growth hormone cDNA, pG23, which I report here.

METHODS

DNA sequence analysis

Sequencing templates for the dideoxy chain terminator technique (Sanger et al., 1977) were prepared by subcloning a PstI partial digest of pG23 into the PstI site of the M13mp8 cloning vector (Messing and Vieira, 1982). The universal sequencing primer (P-L Biochemicals) was used in the individual dideoxy reactions. Deoxyinosine 5'-triphosphate (Sigma) was substituted for deoxyguanosine 5'-triphosphate in some sequencing reactions to resolve compressed areas on the gels.

Chemical Sequencing

The chemical sequencing method of Maxam and Gilbert (1980) was used to analyze two internal regions of the sequence. The 338 base pair PstI fragment of pG23 was prepared for sequencing by purification on a 6% polyacrylamide gel, and then dephosphorylated, kinased, and cleaved with HhaI.

RESULTS

Restriction map and sequencing strategy

A partial restriction map illustrated in Figure 1 reveals the presence of 2 PstI sites internal to the insert of pG23. Thus, digesting the cDNA with PstI would result in 4 fragments; 4.5 kb of pBR322, plus 56, 338, and 446 base pair fragments from the insert. A partial PstI digest was subcloned into M13 and sequenced as shown using the dideoxy method. Ambiguous areas were clarified by examination of multiple gels.

pG23 Sequence

The complete sequence of pG23 is presented in Figure 2. The clone contains 30 nucleotides of 5'-untranslated region, and the entire coding and 3' untranslated regions of growth hormone mRNA. The clone contains 785 nucleotides of sequence, 21 dA-dT residues, and 15 and 13 dG-dC residues at the 5' and 3' ends, respectively. Eight differences between the pG23 sequence and that previously published for another bovine growth hormone cDNA clone (pBP348, Miller et al., 1980) are denoted with asterisks. The three differences found in the 5' untrans-

Figure 1

Sequencing strategy

Restriction sites for the enzymes PstI, PvuII, HhaI and SmaI are shown within the pG23 insert. Open boxes at the PstI ends represent the dG-dC homopolymer tracts introduced during cloning. The solid horizontal lines indicate the direction and extent that each subclone was sequenced by the dideoxy chain terminator method. The dotted lines represent the sequence determined from end labeling the internal PstI fragment of pG23 and chemical sequencing. The total length of this insert including the dG-dC tails and the poly(A) segment is 834 base pairs.

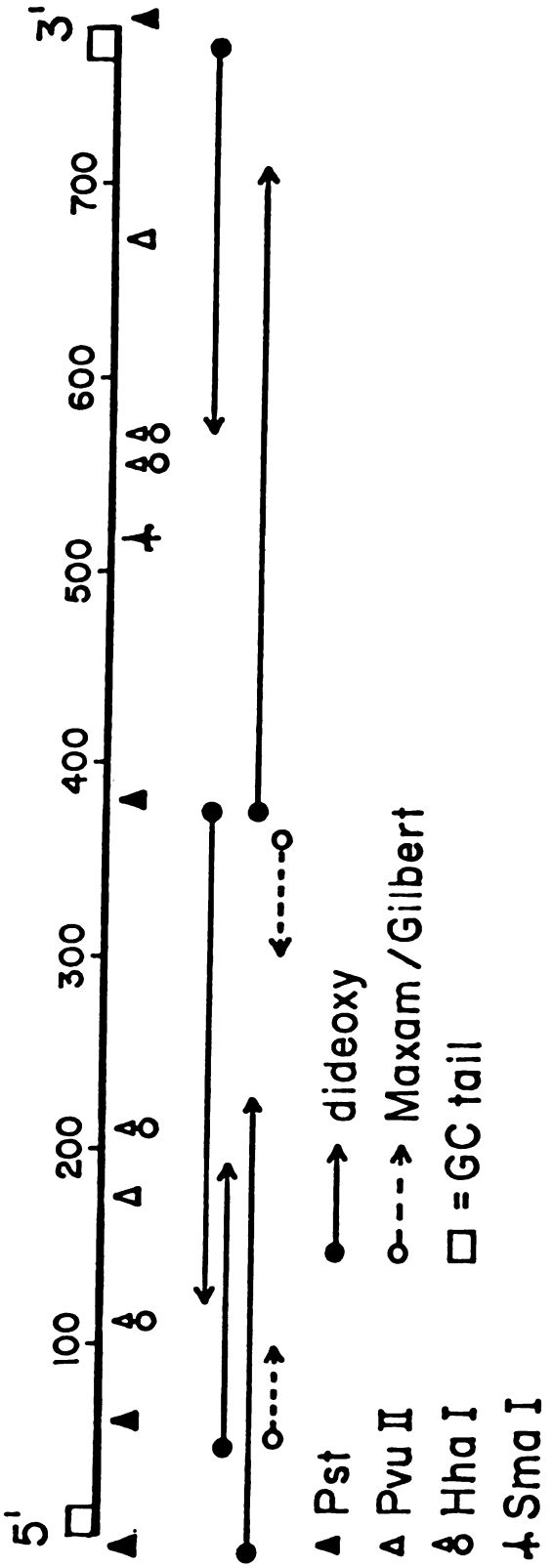
5'
3'
100
200
300
400
500
600
700
dideoxy
Maxam/Gilbert
=GC tail
Pst
Pvu II
Hha I
Sma I

Figure 1

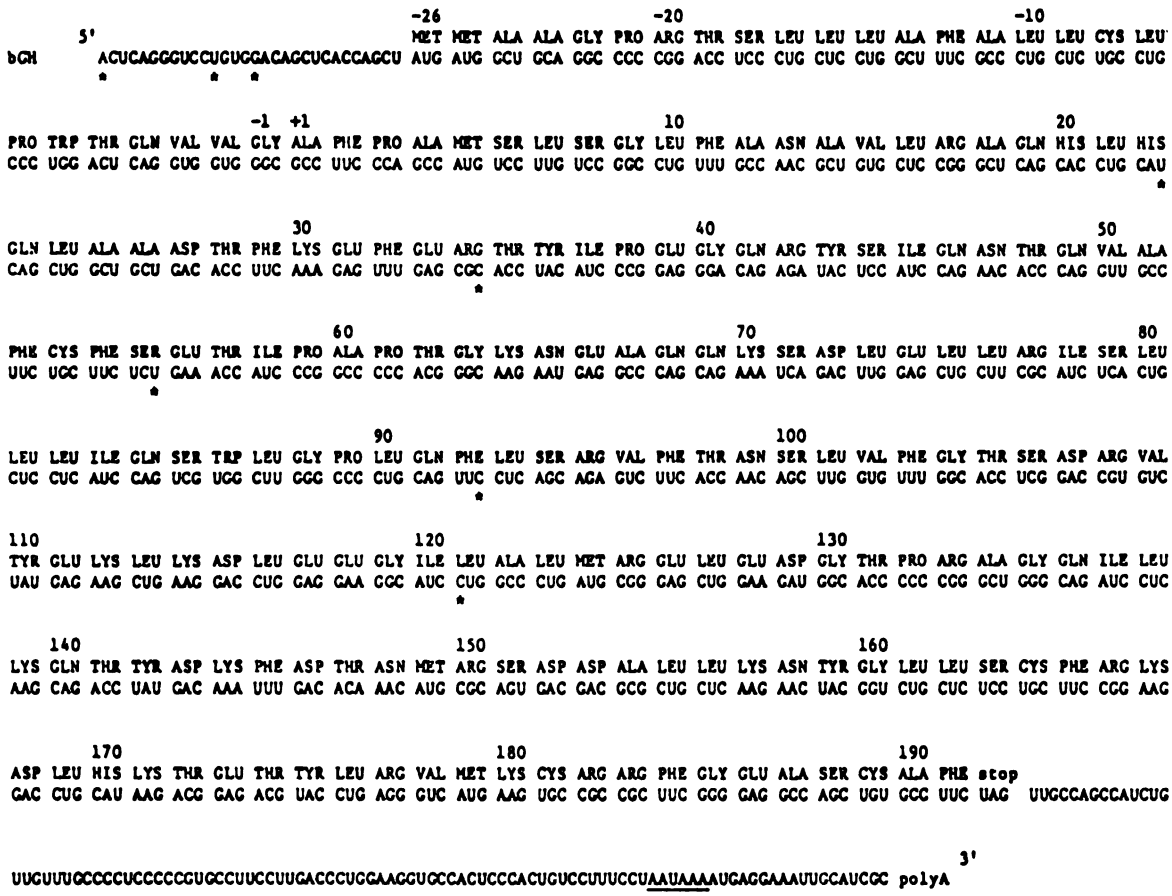

Figure 2

Nucleotide sequence and predicted amino acid sequence of bovine growth hormone mRNA

The amino acid sequence of bovine growth hormone is shown with the mRNA sequence deduced from the clone pG23. Bases marked by an asterisk (*) are nucleotides that differ from pBP348 (Miller et al, 1980).

lated region (Figure 2) include a G to A transition, and the insertion of a T and a G. The remainder of the differences were in the coding region and were silent with respect to the predicted amino acid sequence. A comparison of the codons predicted from the pG23 and pBP348 sequences is shown in Table 1.

DISCUSSION

Bovine growth hormone is known to consist of two allelic variants; one containing valine at amino acid position number 121, and the other with leucine (Seavey et al., 1971). Both our cDNA clone (pG23) and the other cDNA clone (pBP348) are the leucine type, yet there are eight differences in the cDNA sequences. These differences were determined unambiguously in pG23, and matched exactly the sequence of a growth hormone genomic clone isolated by our laboratory (Woychik et al., 1982).

After completing the sequence, we contacted Dr. W. Miller and asked him to verify that these were real differences representing polymorphisms in the growth hormone gene. Further investigation by W. Miller revealed that all but one of the differences we found were probably the result of ambiguities in the sequence of pBP348 (personal communication). The difference in the codon for serine 55 (Table 1) could be due to polymorphism.

Synthesis of the second cDNA strand by self-priming has been shown to result in a high incidence of erroneous bases at the 5' end of a cDNA (Richards et al., 1979). Since the sequence of pG23 matched that of the bovine growth hormone gene exactly, we do not believe that there were any errors in the synthesis of the pG23 cDNA. This

Table 1

Summary of sequence differences in bovine growth hormone mRNA

Amino acid	Position number	Codon in pG23	Codon in pBP348
his	22	CA<u>U</u>	CA<u>C</u>
arg	34	CG<u>C</u>	CG<u>U</u>
ser	55	UC<u>U</u>	UC<u>C</u>
phe	92	UU<u>C</u>	UU<u>U</u>
leu	121	<u>C</u>UG	<u>U</u>UG

The location and identity of the amino acids with codon differences between pG23 and pBP348 are listed.

tendency, however, could explain the errors found in the 5' untranslated region of pBP348. All the other differences are C and T ambiguities, a common problem encountered in the chemical degradation sequencing technique.

In summary, we have established the correct sequence for the bovine growth hormone mRNA, and have found evidence for a single, silent polymorphic variation in the mRNA sequence.

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PART THREE

EFFECT OF STH ON PITUITARY mRNA

ABSTRACT

STH has been shown to be an effective inhibitor of mRNA methylation in bovine pituitary cell cultures. Messenger RNA was labeled with (^{3}H-methyl)-methionine and (^{14}C)-uridine in the presence and absence of 500 μM STH, and digested with RNAse and phosphatase. The products were analyzed by chromatography on urea-sephadex and on HPLC. The results revealed the presence of cap structures lacking 2'-O-methylation and a reduction in internal $m^{6}A$ in mRNA labeled in the presence of STH. STH was shown not to reduce cell viability or protein synthesis. To assess the feasibility of analyzing the methylation of a specific pituitary mRNA, the percent of prolactin and growth hormone mRNAs in (^{3}H)-uridine labeled RNA was quantitated by hybridization of the labeled RNA to complementary DNA clones bound to nitrocellulose.

INTRODUCTION

Most higher eukaryotic mRNAs contain blocked and methylated 5' terminal structures called caps (Rottman et al., 1974). These exist commonly in two forms, m^{7}GpppN'mpN'' (cap 1) and m^{7}GpppN'mpN''mpN (cap 2). Examination of the distrubution of nucleosides in the N' position of the cap reveals a substantial amount of pyrimidine-containing caps, about 20-30% (Kaehler et al., 1979). *In vitro* studies indicate that prokaryotic RNA polymerase II initiates with a 5'-terminal purine triphosphate (Maitra and Hurwitz, 1965), although in some cases pyrimidine triphosphates can be used (Rosenberg and Paterson, 1979). It has been proposed that pyrimidine-containing caps are generated by internal cleavage of nascent transcripts, which are then capped

(Schibler and Perry, 1976), but the subject remains controversial (Perry, 1981). Kaehler et al. showed that treatment of Novikoff hepatoma cells with the methylation inhibitor S-tubercidinylhomocysteine (STH) resulted in a change in the base distribution at the N' position of the mRNA caps (Kaehler et al., 1977; 1979). The shift in distribution favored pyrimidine containing caps in STH treated cells, and was consistent with preferential inhibition of a sub-population of mRNA. The presence of STH may have altered the processing or transport of RNA molecules with internally-generated 5' termini, or altered the expressed mRNA population. These possibilities could not easily be assessed by examination of the bulk mRNA population. We decided to study the post-transcriptional processing and methylation of prolactin and growth hormone mRNAs in primary cultures of bovine anterior pituitary glands. The first step was to characterize the effectiveness of STH in primary pituitary cultures.

In this study, I showed that STH is non-toxic to pituitary cells in culture, and does not alter protein or RNA synthesis significantly. Methylation of mRNA is inhibited internally and at the cap by STH treatment. We planned to determine whether a purified RNA contained a single type of cap under normal conditions, and whether the distribution of cap structures in a specific mRNA shifted when methylation was inhibited. These studies were technically not feasible due to insufficient incorporation of radioisotope into prolactin mRNA.

METHODS

Cell culture and labeling

Bovine anterior pituitary glands were obtained from local

slaughterhouses, minced and dissociated with collagenase (Worthington) and pancreatin (Gibco) essentially as described (Padmanabhan et al., 1982). Monolayer cultures were maintained in Swim's medium (Gibco) in which D-valine was substituted for L-valine to suppress the proliferation of non-lactotroph and non-sommatotroph cell types. The medium was supplemented with 10% dialyzed calf serum (Gibco). STH was the generous gift of Dr. J. K. Coward and synthesized as described (Coward et al., 1977; Kaehler et al., 1977).

Labeling RNA with ^{3}H-methionine and ^{14}C-uridine

For each test condition, media from monolayer cultures was replaced with a minimal volume of Swim's medium with 20% the normal level of methionine. After addition of varying amounts of STH, the cells were incubated 1-3 hours prior to the start of the labeling. Then L-[methyl-^{3}H]-methionine (New England Nuclear, 10-15 Ci/mMol) and (^{14}C)-uridine (Amersham, 450-540 mCi/mMol) were added as indicated in figure legends. After completion of the labeling period, total cellular RNA (Vician et al., 1979) or cytoplasmic RNA (Berger and Birkenmeier, 1979) was prepared. RNA samples were heated to 65^{o}C for 3-5 min., chilled on ice, and adjusted to 0.12 M NaCl, 0.01 M Tris, pH 7.5 for binding to oligo(dT)-cellulose (P-L Biochemicals). Poly(A)$^{+}$ RNA was eluted in buffer containing 0.01 M Tris, pH 7.5, 1 mM EDTA and 0.2% SDS and precipitated with ethanol.

RNAse digestions

Poly(A)$^{+}$ RNA with up to 100 µg of carrier yeast tRNA was digested with 2 units of T_2 RNAse (Sigma) in 40 µl of 5 mM sodium acetate, pH 5, for 3 hours at 37^{o}C. After adding ammonium acetate to 10 mM,

further digestion was achieved by incubation with 0.5 units of bacterial alkaline phosphatase (Worthington) for 1 hour at 37°C. Where indicated, 10 μg of P_1 nuclease (Sankyo) was included in the T_2 digestion.

Separation of cap structure

A DEAE Sephadex column equilibrated in 7 M urea, 0.02 M Tris-HCl pH 7.2, 0.05 M NaCl was used to separate cap structures produced from digestions with RNAse T_2 and alkaline phosphatase essentially as described previously (Kaehler et al., 1979). Samples were eluted with 0.1 to 0.4 M NaCl gradient, fractions collected and counted in a liquid scintillation counter. Oligonucleotides $(pUm)_{1-5}$ were included as standards for the approximate charge eluting across the salt gradient.

Reversed phase HPLC separation

Poly(A)$^+$ RNA samples digested with RNAses T_2 and P_1 and alkaline phosphatase were injected onto a Partisil ODS-5 C-18 reversed phase column (Whatman) and eluted as described previously (Albers et al., 1981). Briefly, elution was isocratic with 0.5% acetonitrile and 0.5 M ammonium formate until m^7GpppGm was eluted. An exponential gradient of 0.5% to 3.0% acetonitrile in 0.5 M ammonium formate was applied until the unmodified adenosine nucleoside eluted and then the gradient was increased to 12% acetonitrile in 0.5 M ammonium formate. Any remaining material was eluted from the column with 20% acetonitrile in water.

Analysis of purine ring labeling

Cells (1.2×10^7 per condition) were incubated with 0 or 10 mM sodium formate and labeled with 0.347 mCi of (^{3}H)-methionine and 0.347

μCi of (^{14}C)-uridine for 24.5 hours. Total poly(A)$^+$ RNA was isolated and an aliquot counted directly. A separate aliquot (2000 cpm) was evaporated to dryness, resuspended in 500 μl of concentrated formic acid, and then heated in sealed glass tubes for 2 hours at 100^{o}C in a sand bath. Labeling of purines was analyzed by chromatography on Partisil 5-SCX (Whatman) with 0.02 M potassium phosphate, pH 3.6 at a flow rate of 0.5 ml/min.

Protein synthesis

Primary cultures of bovine anterior pituitary cells (3 x 10^6 cells per condition) were incubated with 0 or 500 μM STH in Swim's medium containing 10% dialyzed calf serum (Gibco) and 20% the normal level of D-valine (40 μM). Proteins were labeled by addition of L-[3,4(n)-^{3}H] valine (Amersham, 17.5 Ci/mMol) to 0.55 μM for a 2 hour pulse at various times over a 24 hour period. Media was collected after the labeling period, cells were washed with cold phosphate-buffered saline, lysed in 250 μl of 2% SDS and the DNA was sheared with a syringe. Incorporation into protein was determined by precipitation on GF/A filters (Whatman) with trichloroacetic acid and counting the filters in a liquid scintillation counter.

Quantitation by filter hybridization

DNA from hybrid plasmids containing sequences complementary to prolactin or growth hormone mRNA or DNA from the parent plasmid pBR322 was linearized with EcoRI, made 0.33 M in ammonium hydroxide and denatured by boiling 1.5 minutes and quenching on dry ice. The denatured DNA (2.4 μg) was made 2.0 M in NaCl, and spotted on 7 mm nitrocellulose filter circles (Schleicher and Schuell BA83) that had

been premoistened with 0.3 M NaCl and 0.03 M sodium citrate and air dried. After baking 2 hours at 80°C, filters were prewashed in 0.3 M NaCl, 10 mM Tris pH 7.5 and 0.1% SDS for 2 hours at 45°C, and preannealed in buffer containing 0.5 M NaCl, 0.05 M Pipes pH 7.0 (Sigma), 0.4% SDS, 2 mM EDTA, 33% formamide, 0.14 mg/ml nuclease-free tRNA (BRL) and 100 μg/ml poly(A) (P-L Biochemicals).

Filters containing prolactin plasmid pBPRL72 (Sasavage et al., 1982), growth hormone plasmid pG23 (Woychik et al., 1982), or pBR322 (control) were hybridized to several concentrations of (^{3}H)-uridine labeled total RNA for 72 hours at 42°C in the same buffer as for preannealing. Filters were washed once at room temperature and then at 65°C in several changes of 0.15 M NaCl, 0.015 M sodium citrate (1X SSC) and 0.5% SDS. The final wash consisted of 0.075 M sodium chloride, 7.5 mM sodium citrate (0.5X SSC), 0.5% SDS at 65°C. Filters were blotted on 3MM filter paper, placed in scintillation vials, and the bound RNA released with 0.04 M NaOH, neutralized with 0.1 M acetic acid, diluted to 1 ml, and counted with liquid scintillation fluid.

To estimate the percentage of each specific mRNA in the radiolabeled cytoplasmic RNA, counts per minute specifically bound to each filter were plotted as a function of the cpm of RNA present in the hybridization. The percentage of the total represented in a specific message was obtained from the linear portion of the graph and the background binding to pBR322 filters was subtracted.

RESULTS

Demonstration that STH inhibits methylation

To assess the ability of STH to inhibit mRNA methylation in pituitary cells, RNA was labeled with both (^{3}H)-methionine and (^{14}C)-uridine (Table 1). RNA synthesis was apparently inhibited by STH because incorporation of (^{14}C)-uridine into both poly(A)$^{-}$ and poly(A)$^{+}$ RNA was reduced. Methylation was inhibited more than RNA synthesis as indicated by the ratio of ^{3}H to ^{14}C incorporated into RNA. STH inhibited methylation of cytoplasmic poly(A)$^{+}$ RNA by 24% of the control and, in a separate experiment, produced a similar inhibition of total cellular poly(A)$^{+}$ RNA methylation (32%). Thus STH appears to be effective in inhibiting mRNA methylation in pituitary cells.

Existence of cap zero mRNA in STH-treated cells

Normally there is virtually no mRNA in cells of higher eukaryotes that does not bear methylation of the 2' position of the ribose nearest the cap (cap zero). To determine whether STH treatment resulted in the production of cap zero mRNA in pituitary cells, mRNA labeled with (^{3}H)-methionine was digested with RNAse T_2 and alkaline phosphatase and analyzed by chromatography on DEAE-Sephadex (Table 2). This procedure separates the digestion products on the basis of charge. RNAse T_2 is a non-specific nuclease that requires a free 2' hydroxyl on the ribose to hydrolyze the RNA, and therefore subsequent hydrolysis with alkaline phosphatase can produce mononucleotides and cap structures of the following forms: m^{7}GpppN'mpN''mpN, m^{7}GpppN'mpN'', and m^{7}GpppN, where N represents a nucleoside. The results are presented as the percent of the total tritium recovered in

Table 1

Effect of STH on total and cytoplasmic RNA synthesis and methylation

		Control			500 μM STH			
		^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	Inhib
A)	Cytoplasmic Poly(A)$^{+}$ RNA	720	6390	0.113	210	2420	0.086	24
	Cytoplasmic Poly(A)$^{-}$ RNA	9.6×10^{4}	5.6×10^{4}	1.73	3.7×10^{4}	2.6×10^{4}	1.4	19
B)	Total Poly(A)$^{+}$ RNA	7200	9200	0.78	3780	7200	0.53	32
	Total Poly(A)$^{-}$ RNA	1.7×10^{5}	4.2×10^{4}	4.0	6.1×10^{4}	3.0×10^{4}	2.0	50

(A) 8×10^{6} cells were pre-incubated 2 hours in media reduced in methionine, incubated with 0 or 500 μM STH for 1 hour, then labeled for 5 hours with 0.6 mCi (^{3}H-methyl)-methionine and 6 μCi (^{14}C)-uridine. Cytoplasmic RNA was prepared and counted.

(B) 9×10^{6} cells were pre-incubated 1 hour in low methionine medium, incubated with 0 or 500 μM STH for 3 hours, then labeled with 3.2 mCi (^{3}H-methyl)-methionine and 32 μCi (^{14}C)-uridine for 5 hours. Total RNA was prepared and counted.

Table 2

Distribution of methyl label in poly(A)$^+$ RNA

	0 μM STH	500 μM STH
Mononucleosides, m^6A	76	68
Dinucleotides (NmpN)	2	4
Cap Zero (m^7GpppN)	0	1
Cap 1 ($m^7GpppN'mpN''$) and Cap 2 ($m^7GpppN'mpN''mpN$)	22	27

Total poly(A)$^+$ RNA (3000 ^{3}H-cpm) prepared from cells treated with 0 or 500 μM STH as indicated in Table 1 was digested with RNAse T_2 and bacterial alkaline phosphatase and chromatographed on DEAE Sephadex as described in Methods. Results are presented as percent of the total methyl label recovered.

each peak. In the control sample (0 μM STH), radioactivity was distributed into 3 well-separated peaks, mononucleosides and m^6A (uncharged), dinucleotides (NmpN) probably arising from ribosomal RNA contamination (charge -1), and the cap structures, cap one and cap two. The charges on caps one and two are approximately -3.5 and -4.5, respectively. Although the radioactive peak exhibited maxima corresponding to the expected charges, the two were not resolved sufficiently, and are reported as the sum of caps one and two (22% of the total). In the mRNA from cells treated with 500 μM STH, an additional peak of radioactivity was detected at a charge of -2.5, as expected for cap zero structures. This peak represented about 1% of the total tritium and about 3.6% of the total radioactivity in cap structures. Thus STH did result in the appearance in undermethylated cap structures in pituitary cells.

Enrichment for cap zero structures

To study the distribution of nucleosides contained in cap zero structures, it was necessary to increase the radioactivity incorporated into these structures. I decided to try to increase the labeling of cap zero mRNA by extending the labeling time from 5 to 25 hours. Cells pre-incubated with 0 or 500 μM STH were labeled with (^3H)-methionine and (^{14}C)-uridine for 5 or 25 hours and total RNA isolated, fractionated on oligo(dT)-cellulose and counted (Table 3). The ratio of ^3H to ^{14}C incorporated into poly(A)$^+$ RNA was reduced in STH-treated cells compared to control cells, representing approximately 39% inhibition of methylation. The labeled poly(A)$^+$ RNA was digested with RNAse T_2 and alkaline phosphatase, and the products were

Table 3

Effect of STH on RNA methylation in an extended labeling time

	Control			500 μM STH			
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	%Inhib
5 Hour Total poly(A)+ RNA	--	--	--	9060	14100	0.64	--
24 Hour Total poly(A)+ RNA	$1.5x10^{4}$	$9.8x10^{3}$	1.6	$1.8x10^{4}$	$1.9x10^{4}$	0.97	39

For each condition, 1.6×10^{7} cells were incubated 1 hour in media reduced in methionine and then for 2 hours in 0 or 500 μM STH. Subsequently, cells were incubated with 1.4 μCi of (^{14}C)-uridine and 1.6 or 1.25 mCi of (^{3}H-methyl)-methionine for 5 hours (1.6 mCi) or 25 hours (1.25 mCi) when total RNA was isolated, chromatographed on oligo(dT)-cellulose, and counted.

analyzed by chromatography on DEAE-Sephadex using a shallow salt gradient (Table 4). Although in this experiment cap one and two structures were not resolved to baseline, separation was sufficient for estimation of the amount of cap one and cap two present in each sample. The contamination of poly(A)$^-$ RNA was more substantial in this experiment as evidenced by the percent of radioactivity eluting as dinucleosides and trinucleosides (Table 4,A). Therefore, the results have been summarized as percent of the total radioactivity in cap structures (Table 4,B). As expected, no radioactivity in cap zero structures was detected in control RNA samples. Cap zero increased from 8% of the total caps in a 5 hour label to 14% in a 24 hour labeling. The amount of radioactivity present in cap two structures was reduced by STH treatment (34% compared to 18%) but was greater in STH treated cells labeled for 24 hours than in those labeled for 5 hours (18% compared to 24%). It appeared that longer labeling indeed increased the amount of mRNAs bearing cap zero structures.

Distribution of purines and pyrimidines in caps

To ascertain whether STH resulted in a shift in the distribution of purine and pyrimidine-containing caps, samples of RNA from cultures treated with 0 or 500 μM STH and labeled with (^{3}H)-methionine and (^{14}C)-uridine for 24 hours (Table 3) were digested with RNAses T_2 and P_1 and alkaline phosphatase, and the digestion products were separated with a new method (Albers et al., 1981) involving reversed phase HPLC (Table 5). P_1 does not require a free 2' hydroxyl on the ribose for the hydrolysis of mRNA; thus cap two structures are reduced to a core structure exactly the same as that produced by digestion of cap one

Table 4

Distribution of methyl label in poly(A)$^+$ RNA

A	% of Total ^3H	24 hour label		5 hr label
		Control	500 µM STH	500 µM STH
	m^6A, mononucleosides	64.8	56.7	62.9
	Dinucleotides (NmpN)	11.3	27.6	17.2
	Trinucleotides $(Nmp)_2N$	0.48	1.4	1.1
	Cap Zero	0	2.0	1.5
	Cap One	15.4	9.7	15.2
	Cap Two	8.1	2.5	2.2
B	% of total caps	24 hour label		5 hr label
		Control	500 µM STH	500 µM STH
	Cap Zero	--	14	8
	Cap One	66	68	80
	Cap Two	34	18	12

Samples of total poly(A)$^+$ RNA from cultures treated with 0 and 500 µM STH and labeled with (^{3}H-methyl)-methionine and (^{14}C)-uridine as described in Table 3 were digested with RNAse T_2 and bacterial alkaline phosphatase. The digestion products (3700 cpm from cells labeled 24 hours and 5600 cpm from samples labeled 5 hours) were chromatographed on DEAE sephadex as described in Methods, except that the gradient was made more shallow (0.1 to 0.35 M NaCl). Results are presented as percent of the total tritium recovered in each peak (A), and percent of the radioactivity in cap structures (B).

Table 5

Analysis of distribution of methylation by HPLC

Residue	Control (%)	STH (%)
m^7GpppC	0	2
m^7GpppU	0	2.2
m^7GpppG	0	0.8
m^7GpppA and m^7GpppUm	0.6	0.2
m^7GpppCm	6.9	2.6
m^7GpppGm	7.0	4.0
m^7GpppAm	2.9	4.8
m^7Gpppm^{6}Am	14.4	2.6
Cm	2.6	5.0
Um	5.0	2.6
Gm	2.6	7.6
Am	3.0	11.4
m^6A	22.0	10.8
A	24.0	33.0
G	4.2	7.4

The analysis was performed as described by Albers, et al. (1981). Briefly, samples of poly(A)$^+$ RNA (4000 cpm) were digested with T_2 and P_1 RNAses and alkaline phosphatase, evaporated to dryness, resuspended in 20 µl of 0.5% acetonitrile, 0.5 M ammonium formate, mixed with 6 µl of standards for modified and unmodified nucleosides and cap structures, and injected onto a Partisil ODS-5 reversed phase column. Absorbance of standards at 254 nm was monitored and effluent fractions collected and counted. The results are presented as percent of the total tritium counts.

containing mRNAs (m^7GpppNmp), releasing the adjacent methylated nucleotide (Nmp). The solvent system used to elute the digestion products was the same as that described by Albers et al., 1981. All of the possible digestion products were completely separated, except the cap zero structure m^7GpppA and the cap core m^7GpppUm.

Radioactive samples were co-chromatographed with authentic standards. The radioactivity eluting with each peak was summed, and the results are presented as percent of the total tritium counts. In the control sample, a great deal of radioactivity co-eluted with the non-methylated nucleosides adenosine (24%) and guanosine (4.2%). This was increased to 33% and 7.4% respectively in the mRNA from STH treated cells, and probably represents incorporation of (^{3}H-methyl) groups into the purine rings in de novo purine biosynthesis. Radioactivity was distributed amongst the expected cap structures, internal m^6A, and 2'-O-methylated nucleosides released from cap two structures and any contaminating ribosomal RNA.

In order to properly compare the distribution of cap structures in control and STH treated cells, it was necessary to normalize for the number of methyl groups in each residue that could be potentially labeled. These results are presented in Table 6. In control cells, the purine-containing cap predominated, representing 66.1% of the labeled caps. The distribution of caps was altered somewhat in STH treated cells; most notably the U-containing caps increased from 7.1% to 20% of the total caps, at the expense of the purine-containing caps. Caps containing A decreased from 35.3% to 28.2%, and G-containing caps decreased from 30.8% to 24.6% in STH-treated cells.

Table 6

Molar distribution of caps

	Control (%)	STH (%)
m^7GpppC	-	16.2
m^7GpppU	-	18.8
m^7GpppG	-	7.0
$m^7GpppCm$	26.8	11.1
$m^7GpppUm$	7.1	1.2
$m^7GpppGm$	30.8	17.6
$m^7GpppAm$	8.8	20.8
$m^7Gppp m^6Am$	26.5	7.4
C caps	26.8	27.3
U caps	7.1	20.0
G caps	30.8	24.6
A caps	35.3	28.2
Py caps	33.9	47.3
Pu caps	66.1	52.8

Data given in Table 5 has been normalized for the number of methyl groups per residue that could potentially be labeled, and presented as mole percent of cap structures.

Reduction in purine ring labeling

The high percentage of purine ring labeling could possibly be reduced by including sodium formate in the labeling media to dilute the radioactivity in the one-carbon precursor pool. The inclusion of up to 20 mM sodium formate did not reduce cell viability as evidenced by unaltered trypan blue exclusion. Labeling cells with (^{3}H)-methionine and (^{14}C)-uridine resulted in a reduction of the ratio of ^{3}H/^{14}C label in poly(A)$^{+}$ RNA from the control value of 0.97 to 0.74 in cultures treated with 10 mM sodium formate. This reduction could be due to less incorporation of tritium into purine rings in the presence of sodium formate. The labeled RNA was subjected to acid hydrolysis to release purine bases, which were subsequently separated on cation exchange HPLC. Conditions were selected for separation of standards for m^{7}G, G, A, and m^{6}A after testing several salts at various concentrations and pH. Labeled guanine (G) eluted too close to the peak of labeled apurinic RNA to get an accurate measurement of the extent of ring labeling of G. However, m^{7}G, A, and m^{6}A were well resolved in the labeled RNA. Control samples showed extensive ring labeling of adenosine, as expected, however none was detected when 10 mM formate was used (data not shown). The ratio of m^{6}A to m^{7}G was 3.4/1 in the poly(A)$^{+}$ RNA samples from cells treated with 10 mM formate.

Effect of STH on protein synthesis

To ensure that STH treatment did not alter cellular metabolism, protein synthetic capability was monitored by pulse-labeling of proteins with (^{3}H)-L-valine (Table 7). Trichloroacetic acid (TCA) precipitable radioactivity incorporated into proteins during 2 hour

Table 7

Incorporation of radioactive amino acid into protein

Time (Hrs)	0 μM STH	500 μM STH
3-5	3.6×10^5	3.3×10^5
4-6	2.4×10^5	2.2×10^5
7.5-9.5	3.6×10^5	2.3×10^5
20.5-21.5	3.1×10^5	3.3×10^5
23-25	1.6×10^5	2.0×10^5

Proteins were labeled with (^{3}H)-L-valine for 2 hour pulses after incubation with 0 or 500 μM STH as described in Methods. The results are presented as total trichloroacetic acid-precipitable counts obtained at various times after addition of STH.

pulses over a 24 hour course of incubation with STH revealed essentially no difference in protein synthesis in STH treated cells. The total incorporation ranged from 1.6 x 10^5 to 3.6 x 10^5 cpm, however this resulted from inconsistent amounts of cells plated rather than differences in protein synthesis or inaccurate TCA precipitation.

Estimation of prolactin and growth hormone content in labeled cytoplasmic RNA

Efficient labeling of poly(A)$^+$ RNA is necessary to obtain sufficient labeled prolactin or growth hormone mRNA for methylation analysis. RNA was labeled with (^{3}H)-uridine over a 24 hour period in cells pre-incubated with 0 or 500 μM STH (Table 8). Cytoplasmic RNA was extracted and an aliquot counted. Incorporation was similar for control and STH treated cultures and increased over time in close to a linear fashion for each. The content of prolactin and growth hormone mRNA in samples labeled for 24 hours was determined by hybridization to cloned complementary DNA sequences bound to nitrocellulose filters. The specific binding of prolactin and growth hormone mRNA over the background binding to filters containing pBR322 was 3.2 and 1.7 fold, respectively. The results indicated that prolactin and growth hormone constitute 0.012 and 0.0035% of the labeled cytoplasmic RNA, respectively.

In an attempt to increase the utilization of (^{3}H)-uridine for labeling RNA, 20 mM glucosamine hydrochloride was added 1 hour prior to labeling with (^{3}H)-uridine. In contrast to results obtained with mammary cell cultures (Guyette et al., 1979), this resulted in a reduction rather than a stimulation of radioactive uridine incor-

Table 8

Rate of accumulation of ^{3}H-uridine into total RNA

Time (Hrs)	0 μM STH	500 μM STH
2	2.1	3.7
5	9.3	8.1
10	20	16
20	25	22
24	36	42

Two plates with 5×10^{6} cells each were incubated with 0 or 500 μM STH for 3 hours and labeled with 2.3 μM (5,6-^{3}H)-uridine (Amersham, 43 Ci/mMol). At various times after the addition of the isotope, cells were washed in cold phosphate-buffered saline and cytoplasmic RNA was prepared separately from each dish (Berger and Birkenmeier, 1979). Results are reported as an average total incorporation of radioactivity into cytoplasmic RNA (cpm $\times 10^{-6}$).

porated into RNA (data not shown).

DISCUSSION

Initial experiments indicated that STH is effective in inhibiting RNA methylation in both poly(A)$^-$ and poly(A)$^+$ RNA populations in monolayer cell cultures of bovine pituitary cells. Also, STH treatment may result in a diminution of RNA synthesis. In several experiments, the difference in (^{14}C)-uridine incorporation in control and STH treated cells was variable, probably due to differences in efficiency of RNA extraction; however, the STH treated cells usually incorporated less. The extent of inhibition as judged by the ratio of (^{3}H)-methionine to (^{14}C)-uridine incorporated into RNA appeared to be 20 to 30% in both total RNA and cytoplasmic RNA (Table 1). Examination of the distribution of methyl label in cap zero, one, and two structures (Table 2, 4) revealed the presence of cap zero structures only in mRNA from STH treated cells. The distribution between mononucleosides and cap structures was almost identical to that reported previously for STH treatment of Novikoff hepatoma cells (Kaehler et al., 1977); cap zero represented 5.3% of the methyl label in Novikoff cells, and only 1% in pituitary cells. Importantly, the Novikoff cells were treated with 250 μM STH instead of 500 μM and labeled for one hour instead of 5 hours. This difference probably reflects variable sensitivity to inhibition of methylation by STH and a slower metabolism in pituitary cells.

In an attempt to enhance the amount of label in cap zero structures, pituitary primary cultures were treated with STH and the labeling time increased from 5 to 24 hours. This longer labeling time

increased the radioactivity recovered in cap zero structures from 8% of the tritium labeled caps to 14% (Table 4).

The amount radioactivity in of cap two structures was decreased in STH treated cells compared to the control, but appeared to be slightly greater when cells were labeled for 24 hours rather than 5 hours. Although it is possible that STH loses its effectiveness over a long period of time in the cell, it is not a substrate for SAH hydrolase (Chiang et al., 1977) and has been shown to be a metabolically stable compound in neuroblastoma cells (Crooks et al., 1979). More likely is the possibility that the difference is related to differential kinetics of labeling. Methylation of the N'' position resulting in a cap two structure occurs cytoplasmically (Perry, 1981). Subsequent experiments incorporated a 24 hour labeling period because this allowed for more efficient utilization of radioisotope as well as production of slightly more labeled cap zero RNA.

A new high performance liquid chromatography system was utilized to separate all the methyl labeled nucleosides and cap structures produced by digestion with RNAses T_2 and P_1, and alkaline phosphatase. The separation of all the possible cap structures in a single step permitted accurate quantitation of molar amounts of each cap in the labeled mRNA. The results of the HPLC analysis (Table 5) revealed a significant amount of tritium label in purine rings, the extent of which was increased when the methylation inhibitor was present. This finding is important because it underlines the weakness of estimating the degree of inhibition of methylation by the ratio of (^{3}H)-methionine to (^{14}C)-uridine incorporated. Internal m^6A methylation was

markedly inhibited (approximately 50%) as was m^6A contained in cap structures (65% reduction). Presentation of the results as mole percent of each type of cap structure (Table 6) showed that 42% of the caps were cap zero form in STH-treated cells whereas the amount of cap zero present appears much less if taken as a strict percentage of the total tritium incorporated (Table 4,5). Thus, the HPLC technique presents a rapid, simple, and more accurate means of determining the distribution of cap structures in a mRNA sample.

The shift in distribution of caps following STH treatment of cells resulted in an increase in uridine-containing caps at the expense of those containing purine (Table 6). The percent of the radioactivity represented in a specific cap can vary by as much as 5% of the total in samples similarly prepared and analyzed by HPLC. Although documentation of a shift in distribution would necessitate several analyses, it seems possible that the increase from 7.1% uridine containing caps in the control RNA to 20% in RNA from STH treated cells represents a real increase.

Kaehler et al. (1979) reported that cap one structures containing uridine in the N' position increased in cytoplasmic RNA from Novikoff hepatoma cells when the cells were treated with STH. Considering that greater than 50% of the cap structures were of the cap zero form in STH-treated cells, this result is misleading. If the distribution of nucleosides is calculated for the sum of cap zero and cap one structures on a molar basis, uridine-containing caps represent 14.5% and 13.2% of the total caps in control and STH-treated cells, respectively. Perhaps U-containing caps were less sensitive to inhi-

bition of 2'-O-methylation by STH. If this were the case, the percent of the radioactivity in cap structures containing uridine would increase, but the total molar amount of uridine caps would not.

The potential increase in uridine at the N' position of the cap in mRNA from pituitary cells treated with STH could arise by several mechanisms:

1) The cell could respond to inhibition of methylation by inducing or increasing the production of certain mRNAs that happen to contain uridine in the N' position of the cap.
2) Inhibition of methylation could increase the processing, transport or stability of a group of mRNA molecules containing uridine in the N' position of the cap.
3) STH could be interfering with initiation of transcription, causing an increase in initiation at uridine, or an increase in mRNAs whose termini are generated by internal cleavage of primary transcripts.

We felt that these issues could be clarified through study of the processing and methylation of specific mRNAs such as prolactin or growth hormone.

Having shown that STH is effective for inhibition of methylation of mRNA internally and at the cap site, several preliminary experiments were required before embarking on analysis of methylation patterns in specific mRNA molecules. These included demonstrating that the methylation inhibitor is non-toxic to pituitary cells, elimination of purine ring labeling, and estimation of the percent prolactin mRNA and growth hormone mRNA in radiolabeled pituitary RNA.

Purine ring labeling was completely blocked by addition of 10 mM sodium formate to the labeling medium, facilitating quantitation of the extent of methylation inhibition by $^{3}H/^{14}C$ ratios. Cell viability was monitored in control cells and STH treated-cells by ability to exclude trypan blue; there was no difference in viability. Protein synthetic capability was also not impaired in the presence of STH, as indicated by the ability to incorporate equal amounts of radioactive amino acid into the protein after 24 hours of incubation with 0 or 500 μM STH (Table 7). Thus, STH is apparently a non-toxic methylation inhibitor in pituitary monolayer cultures.

A time course of incorporation of (^{3}H)-uridine into RNA revealed very little difference in total RNA synthesis in control and STH treated cells (Table 8). These results are more reliable than (^{14}C)-uridine incorporation reported in Tables 1 and 3 because the efficiency of extraction was improved in the former case, in addition to the fact that values from two separate extractions were averaged to obtain those results. When the cytoplasmic RNA was analyzed for poly(A) content, it appeared that the cytoplasmic appearance of tRNA, rRNA and mRNA were differentially sensitive to STH, however the data was not sufficiently clear for further conclusions to be drawn (data not shown).

Filter hybridization analysis of RNA labeled 24 hours with (^{3}H)-uridine indicated that prolactin mRNA represents 0.012% of the labeled cytoplasmic RNA, whereas growth hormone mRNA represents 0.0035%. Approximately 1.3% of the cytoplasmic RNA could be bound to oligo(dT)-cellulose. On the basis of these estimates, it would be possible to

measure the rate of accumulation of prolactin or growth hormone mRNA, but it would be technically quite difficult to analyze the methylation pattern of either RNA. For instance, labeling 2.9×10^8 cells with 9.5 mCi of (^{3}H)-methionine for 24 hours yields only 1.2×10^5 cpm of poly(A)$^+$ RNA. Assuming that the amount of either mRNA present in (^{3}H)-uridine labeled RNA is proportional to the amount in (^{3}H)-methionine labeled RNA, less than 50 cpm would be present in prolactin specific sequences. An accurate analysis by HPLC would ideally utilize about 2000 cpm; thus it is not feasible to analyze the methylation pattern of either message with this approach. The steady-state level of prolactin mRNA in pituitary glands is approximately 50% of the poly(A)$^+$ RNA. Therefore, it is possible that a methylation analysis could be done on <u>in vitro</u> labeled RNA (Malek et al., 1981), but not on RNA labeled in cell cultures.

The prolactin gene is probably transcribed at a relatively low level (about 0.04-0.065% ; H. Meisner, unpublished results), possibly explaining the low abundance of prolactin in (^{3}H)-uridine labeled RNA. The abundance of prolactin message in the steady-state population of mRNA in the pituitary gland must result from a long half-life.

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PART 4

EFFECT OF STH ON HeLa CELL mRNA HALF-LIFE AND CYTOPLASMIC APPEARANCE

ABSTRACT

S-tubercidinylhomocysteine (STH) is a structural analog of S-adenosylhomocysteine and a potent inhibitor of S-adenosylmethionine-dependent methyltransferase reactions. We have investigated the effects of STH on HeLa cell mRNA metabolism. Labeling HeLa cell RNA with (^{3}H-methyl)-methionine and (^{14}C)-uridine reveals a slight inhibition of mRNA synthesis with a striking inhibition of methylation of the mRNA. mRNA synthesized in the presence of 0, 50, and 500 μM STH was digested with RNases T_2 and P_1, and alkaline phosphatase. The modified nucleosides and cap cores were analyzed by HPLC on ion exchange and reversed phase matrices. The results indicated that internal m^6A was reduced by 65% at 50 μM and about 83% at 500 μM. The m^6A contained in cap structures was similarly reduced at both concentrations of STH. At the higher level of STH, substantial amounts of cap structures lacking 2'-O-methylated nucleosides (m^7GpppN, cap zero) were detected. To test the possibility that methylation affects mRNA stability, mRNA half-life was measured with a (^{3}H)-uridine pulse-chase experiment. The half-life of undermethylated mRNA was unchanged compared to the control. To determine whether mRNA methylation is coupled to nuclear processing or transport, the time of cytoplasmic appearance of poly $(A)^+$ RNA in STH treated cells was compared to control mRNA. The results indicated a significant lag in the time of appearance of the poly$(A)^+$ RNA, suggesting that mRNA methylation may be required for efficient processing or transport.

INTRODUCTION

Since the discovery that eukaryotic mRNA is capped and methylated (Rottman et al., 1974), functions have been suggested for some of these post-transcriptional modifications. Capping may be important for protecting the mRNA against nucleolytic attack (Furuichi et al., 1977; Melton et al., 1980), and methylation in the cap (m^7G) has been shown to function in enhancing the binding of mRNA to 40S rRNA subunits (Both et al., 1975). 2'-O-methylations of the ribose moieties have been shown to influence the translatability of mRNAs, albeit at a much less dramatic level than m^7G (Muthukrishnan et al., 1978). There may be additional functions for 2'-O-methylation that have not yet been discovered.

Messenger RNA of higher eukaryotes is more methylated than that of lower eukaryotes; yeast and some plant mRNAs are devoid of internal m^6A (Banerjee, 1980). It has been suggested that there must be a function for m^6A since it is more abundant in more complex organisms. Internal m^6A has been found within the sequences G-m^6A-C and A-m^6A-C (Wei and Moss, 1977; Nicholas and Welder, 1981), perhaps indicating some specificity in the methylase that catalyzes this modification. Attempts to determine whether m^6A is conserved in the processing of mRNA precursors have produced conflicting results (Lavi and Shatkin, 1975; Sommer et al., 1978), but in the case of SV40, m^6A clearly occurs both within and outside of intervening sequences (Aloni et al., 1979). Thus, a simple model for the potential role of m^6A in splicing has not emerged.

Studies on viral systems provide some indication that m^6A methyl-

ation is altered in infected cells and important for maturation of viral transcripts. Infection of human epidermoid carcinoma cells (HEp-2) by herpes virus results in a shut-off of host m^6A methylation (Bartkoski and Roizman, 1978), but the reason for this perturbation is still unknown. Internal m^6A methylation has been implicated in the splicing of avian sarcoma virus transcripts in experiments where cycloleucine, an inhibitor of S-adenosylmethionine synthetase, was utilized to inhibit methylation (Stoltzfus and Dane, 1982).

If internal m^6A plays an important role in the metabolism of viral RNA, it is important to determine whether it serves a similar function in the maturation of non-viral transcripts. It is known that m^6A methylation is not essential for processing or transport because normal sized undermethylated mRNA can be found in the cytoplasm of cells when methylation inhibitors are employed (Kaehler et al., 1977). Bachellerie et al. (1978) suggested that methylation might be important for the efficient processing of non-viral transcripts based on a transient shift of pulse-labeled nuclear RNA to higher molecular weight in cycloleucine treated BHK cells.

Methylation may be involved in several stages of mRNA metabolism, however no evidence suggests that internal m^6A methylations play a role in mRNA translation. In fact, hypermethylated SV40 mRNA is translated <u>in vitro</u> at the same efficiency as control mRNA (Kahana et al., 1981), and undermethylated RNA is incorporated into polysomes at an apparently normal rate (Dimock and Stoltzfus, 1979). Thus, it remains unclear exactly what the role of m^6A methylation is in mRNA metabolism.

We have used the methylation inhibitor, S-tubercidinylhomocysteine (STH, Figure 1), to further probe the function of 2'-O-methylation and m^6A methylation in HeLa cell mRNA metabolism. STH is a competitive inhibitor of mRNA methyltransferases due to its structural similarity to SAH. Previous studies have indicated that the compound, STH, is stable in cells since it is not a substrate for the SAH hydrolase (Chiang et al., 1977; Crooks et al., 1979). Treatment of cells with STH results in subtle changes, if any, in rRNA methylation, marked reduction in some tRNA methylations (Rottman et al., 1979; Crooks et al., unpublished results). Although STH does inhibit the (guanine-7)-methyltransferases of Newcastle Disease virus and vaccinia virus *in vitro*, it apparently did not inhibit m^7G formation in Novikoff cells (Kaehler et al., 1979).

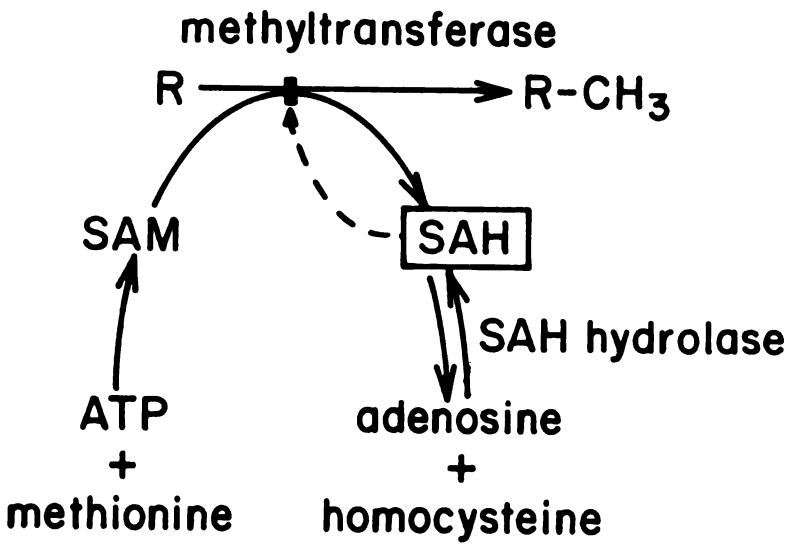
In the present study, we have demonstrated the efficacy of this inhibitor for blocking 2'-O-methylation and m^6A methylation in HeLa cells. STH is a particularly useful tool for probing the function of mRNA methylation in metabolism because it is not cytotoxic and does not inhibit protein synthesis. We investigated the effect of undermethylation on the cytoplasmic stability of mRNA and did not detect any alteration. The time of appearance of undermethylated RNA in the cytoplasm was also monitored, and the results indicated that undermethylation delays the release of mRNA from the nucleus. The delay in cytoplasmic entry suggests that methylation may be required for efficient processing or transport of HeLa cell mRNA.

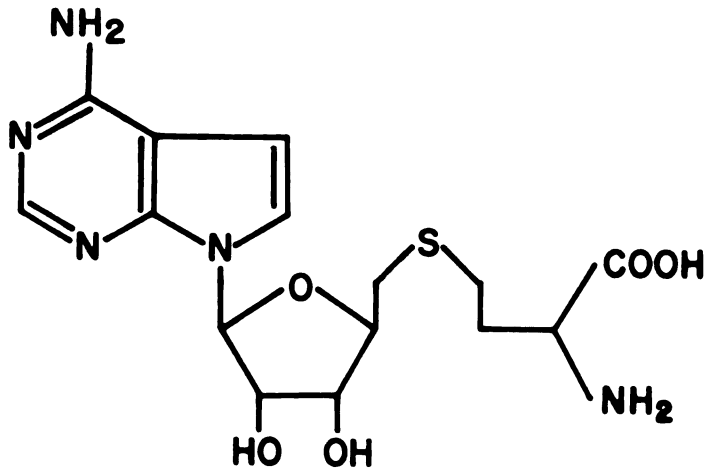
Figure 1

Structure of STH and site of action

S-adenosylmethionine synthetase catalyzes the synthesis of S-adenosylmethionine (SAM) from methionine and ATP. SAM serves as the methyl donor for many cellular methylation reactions producing S-adenosylhomocysteine (SAH). The concentration of SAH is normally low in the cell; it is metabolized by the action of SAH hydrolase which degrades SAH to adenosine and homocysteine. SAH is a competitive inhibitor of many SAM-dependent methyltransferases.

The structure of STH is shown at the right.





STH
7-Deaza SAH

Figure 1

MATERIALS AND METHODS

Cell treatment

HeLa cells were maintained in spinner cultures in Joklik's modified Eagle's medium (MEM) in 10% horse serum. For the duration of all experiments, media was supplemented with 15 mM Hepes, pH 7.4 and 2 mM glutamine. STH was the generous gift of James K. Coward and synthesized as described (Coward et al., 1977; Kaehler et al., 1977). Stock solutions of STH were prepared fresh in 10mM HCl. Control cultures were treated with an equal volume of 10 mM HCl.

RNA isolation

Cells were washed in phosphate buffered saline and incubated 10 minutes on ice in buffer containing 10 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM magnesium acetate. Brij-58 and sodium deoxycholate were each added to a final concentration of 0.24% to lyse the cells. Nuclei were removed by centrifugation. Poly(A)$^+$ RNA was prepared by two passes of cytoplasmic extracts over oligo(dT)-cellulose (Collaborative Research) in 0.5 M LiCl, 10 mM Tris-HCl, pH 7.5, 0.5% SDS and eluted in the same buffer without LiCl. The RNA was heated at 95^o C for 5 minutes and quenched on ice before the second binding to oligo(dT).

RNA digestions

Poly(A)$^+$ RNA containing 50-100 µg of yeast tRNA (BRL) was digested with 2 units of T_2 RNase (Calbiochem) and 10 µg of P_1 nuclease (Sankyo) in 40 µl of 5 mM sodium acetate, pH 5, for 3 hours at 37^o C. After adding ammonium acetate to 10 mM, further digestion was achieved by incubation with 0.5 units of bacterial alkaline phosphatase

(Worthington) for 1 hour at 37° C.

HPLC separation

Normal and modified nucleosides and cap structures were separated by chromatography on a Brownlee-SAX anion exchange column connected in series to a Partisil ODS-3 5 micron reversed phase column (Whatman) in a procedure similar to the one previously described (Albers et al., 1981). Separation of non-charged residues was achieved with a linear gradient of H_2O and 10% acetonitrile in H_2O which was made exponential after the elution of adenosine. Following the elution of $m^6{}_2A$, the system was washed with H_2O and the charged residues were eluted with isocratic 1 M ammonium formate. Upon elution of m^7GpppC (C_o), a linear gradient of 1 M ammonium formate and 10% acetonitrile in 1 M ammonium formate was applied. After elution of $m^7GpppGm$ (G_1) the gradient was made exponential.

RESULTS

STH dose curve for HeLa cells

To optimize the concentration of STH required to inhibit mRNA methylation in HeLa cells, cultures were treated with concentrations of STH ranging from 50 μM to 1 mM and labeled with (^{3}H-methyl)-methionine and (^{14}C)-uridine. (Figure 2). There appeared to be a slight reduction in mRNA synthesis at all concentrations of STH tested, as judged by the lower incorporation of (^{14}C)-uridine into poly(A)$^+$ RNA. Incorporation of (^{3}H)-methionine into mRNA indicated that methylation was reduced more than synthesis at 50 μM STH and appeared to be maximally reduced at 500 μM.

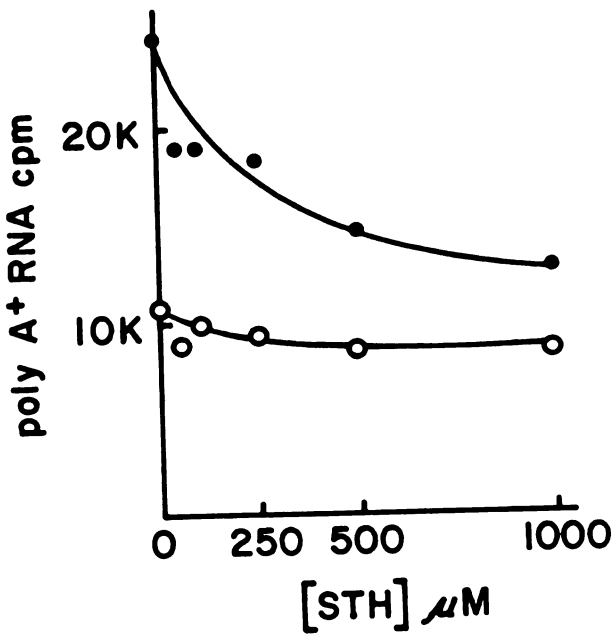


Figure 2

STH dose curve for inhibition of poly(A)$^+$ RNA methylation

4 x 10^6 cells were treated with varying amounts of STH in methionine-free Joklik's MEM supplemented with 5% dialyzed horse serum and 20 mM sodium formate. After 45 minutes, actinomycin D was added to 40 ng/ml to inhibit the synthesis of rRNA. Cells were labeled for 4 hr with (^{3}H-methyl)-methionine (0.15 mCi/ml, 12 Ci/mmole) (•-•) and (^{14}C)-uridine (0.1 μCi/ml, 0.48 Ci/mmole) (o-o). Cytoplasmic poly(A)$^+$ RNA was isolated as described in Methods.

At all concentrations of STH tested, there was no change in cell viability, based on trypan blue exclusion. In a separate experiment, cells were treated with 0 and 500 μM STH for 10 hours, then proteins were pulse-labeled for 2 hours with (^{35}S)-methionine. Examination of the radioactive proteins on one dimensional polyacrylamide gels indicated that protein synthetic capability was unaltered by STH treatment (data not shown).

Quantitation of cap distribution and inhibition of methylation

To determine the extent of inhibition of the various nucleoside residues, mRNA was digested with RNases T_2 and P_1 and alkaline phosphatase, and the digestion products separated on HPLC. RNase T_2 is a nonspecific ribonuclease that cleaves RNA with free 2' hydroxyl groups to produce nucleotides with 3' phosphates and cap cores (e.g. m^7GpppN'mpN''mpNp). Cleavage by nuclease P_1 is not blocked by the presence of a methyl group on the 2' position of the ribose and thus reduces cap two structures to a core (m^7GpppNmp) and releases the 2'-O-methylated nucleotide. After simultaneous digestion with T_2 and P_1, the cap cores and modified and unmodified nucleotides were dephosphorylated with alkaline phosphatase. The HPLC separation employed was developed for rapid, simple quantitation of modified nucleosides and cap structures in a single chromatographic step. All of the possible core cap structures, unmodified nucleosides, 2'-O-methylated nucleosides, base methylated nucleosides including m^5C, m^6A, and $m^6{}_2$A, can be cleanly separated (Figure 3).

The cap structures are charged and retained on the anion exchange pre-column while the non-charged nucleosides are separated as they are

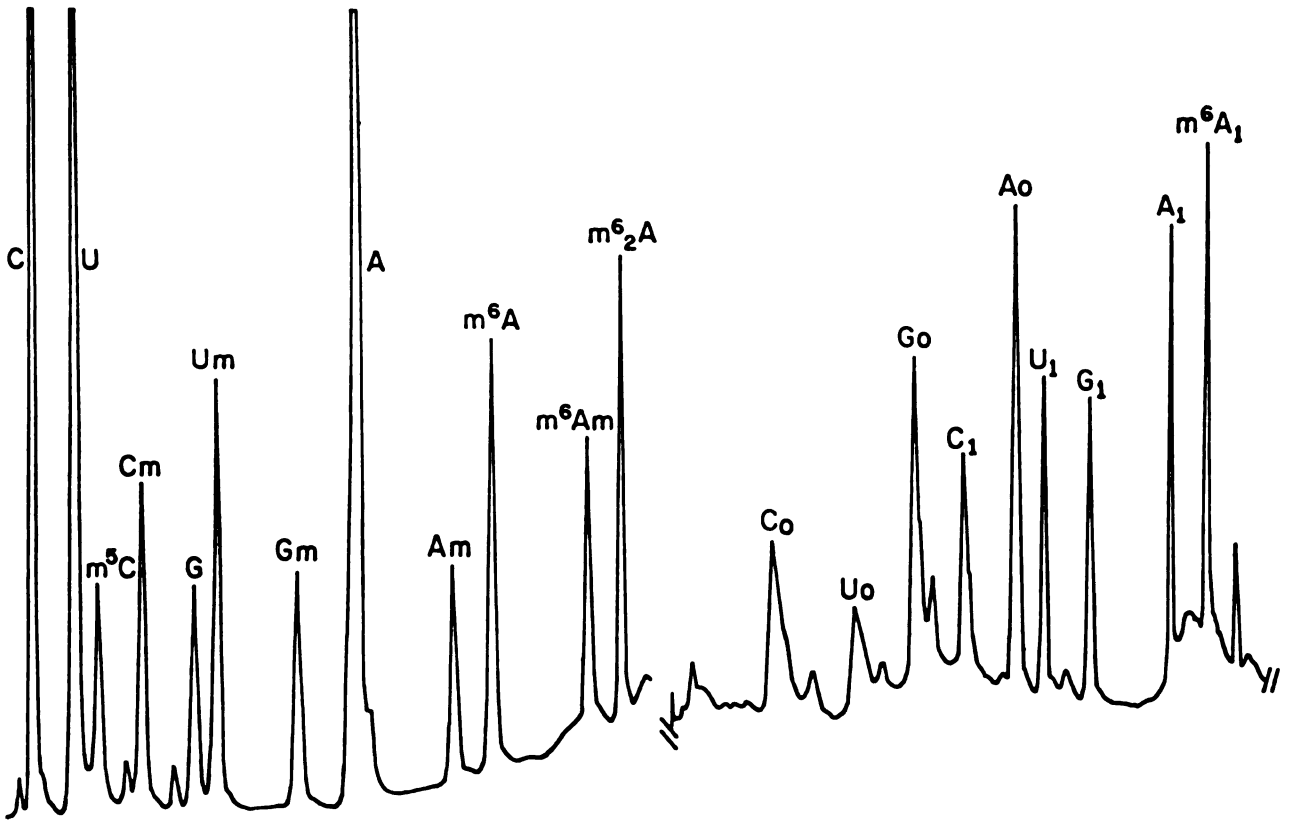


Figure 3

Method for separation of modified and unmodified nucleosides, and cap structures

A typical absorbance profile of authentic standards separated on tandemly-linked Brownlee-SAX anion exchange column and Whatman Partisil ODS 3 reversed phase column is shown. Elution was accomplished as described in methods.

eluted through the reversed-phase C-18 column with acetonitrile. Subsequently, nine different cap cores are separated by elution with ammonium formate and acetonitrile.

Labeled RNA was analyzed in this fashion by co-chromatographing it with authentic standards. Fractions were collected and counted. There was very little incorportation of methyl label into the rings of adenine and guanosine (less than 4% of the total methyl label), and no m^5C was detected in any of the samples analyzed. A trace of m^6Am was only detected in the control poly(A)$^+$ RNA. Surprisingly, $m^6{}_2A$ was detected in each sample. The amount present was not consistent with ribosomal RNA contamination, however the quantities were sufficiently small that it was not pursued further.

The radioactivity in each peak was divided by the number of methyl groups labeled and normalized to the total amount of cap structures recovered. The results are presented as percent inhibition compared to the control, untreated cells (Table 1). In the control sample, the ratio of m^6A per cap was approximately 3.4 to 1. This was reduced by 65% with 50 μM STH and by 83% with 500 μM STH. In control cells, caps bearing m^6A in the N' position (m^7Gpppm^6Am) represented 31 percent of the total, on a molar basis. This was reduced by 58 and 88% in cells treated with 50 and 500 μM STH, respectively. The total amount of adenine containing caps was similar in all cases, ranging from 38 to 27%.

No cap zero containing mRNAs (m^7GpppN, No) were detected in control mRNA samples. However, STH treatment resulted in a trace of mRNA lacking 2'-O-methylation (cap zero) at 50 μM (7%) and substantial

Table 1

Percent inhibition of mRNA methyation by STH

	50 μM STH	500 μM STH
Internal m^6A	65	83
$m^7Gppp\underline{m}^6\underline{A}m$	58	88
$m^7GpppN'\underline{m}pN''(m)$	7	48
$m^7GpppN'mpN''\underline{m}$	50	60

Cytoplasmic HeLa cell poly(A)$^+$ RNA labeled with (^{3}H-methyl)-methionine and (^{14}C)-uridine (Figure 2) was digested with RNase T_2, nuclease P_1, and alkaline phosphatase. The digestion products were co-chromatographed with standards and separated by HPLC as described in methods. Fractions of 0.6 ml were collected and counted. The inhibition of methylation was calculated by dividing the radioactivity eluting in each peak by the number of methyl groups that could potentially be labeled in the eluted compound and then normalizing to the total radioactivity recovered in cap structures. The results are presented as the percent inhibition compared to the control value (0 μM STH). The position of the methylation that was deficient in STH-treated cells is underlined.

amounts at 500 μM (48%). 2'-O-methylation of the N'' position to produce cap two structures was inhibited by 50% with 50 μM STH and by 60% with 500 μM STH.

STH effect on mRNA stability

Knowing that STH treatment resulted in inhibition of 2'-O-methylation at the cap and m^6A methylation both internally and in the cap, we investigated the effect of undermethylation on mRNA stability. The turnover of poly(A)$^+$ RNA was measured by treating cells with 0, 50, and 500 μM STH, pulse labeling cells with (^{3}H)-uridine, and then chasing in the presence of unlabeled uridine and cytidine. It is essential that the mRNA be completely free of rRNA or the measured half-life can be artificially long, therefore we chromatographed each RNA sample twice on oligo(dT)-cellulose. Regardless of the concentration of STH used to pretreat the cells, the mRNA exhibited a major half-life component of about 10.5 hours (Figure 4). Similar results were obtained when actinomycin D was used to inhibit rRNA synthesis (data not shown). Thus, undermethylation did not dramatically alter the stability of the mRNA in the cytoplasm when examined as a mixed population at concentrations of STH that almost completely inhibit m^6A methylation and reduce 2'-O-methylation 50 to 60%.

Effect of STH on nuclear events

Although mRNA methylation is apparently not necessary for processing or transport since undermethylated poly(A)$^+$ can be found in the cytoplasm, it may nonetheless influence the kinetics of nuclear RNA metabolism. We reasoned that if undermethylation influenced the rate of processing and/or transport of the mRNA, there would be a lag

Figure 4

Turnover of poly $(A)^+$ RNA

HeLa cells were concentrated to 2 x 10^6 per ml and pretreated with 0, 50 μM or 500 μM STH for one hour. Medium was then made 0.125 μM in (5,6-^{3}H)-uridine and cells were labeled 2.5 hours. The medium containing radioactive uridine and STH was removed by centrifugation of cells through phosphate buffered saline. Cells were resuspended at 3.5 x 10^5 cells per ml. in medium 10 mM in uridine and 5 mM in cytidine. 4 x 10^6 cells were removed at various times and cytoplasmic poly $(A)^+$ RNA isolated. Control (●-●), 50 μM STH (x-x), and 500 μM STH (o-o).

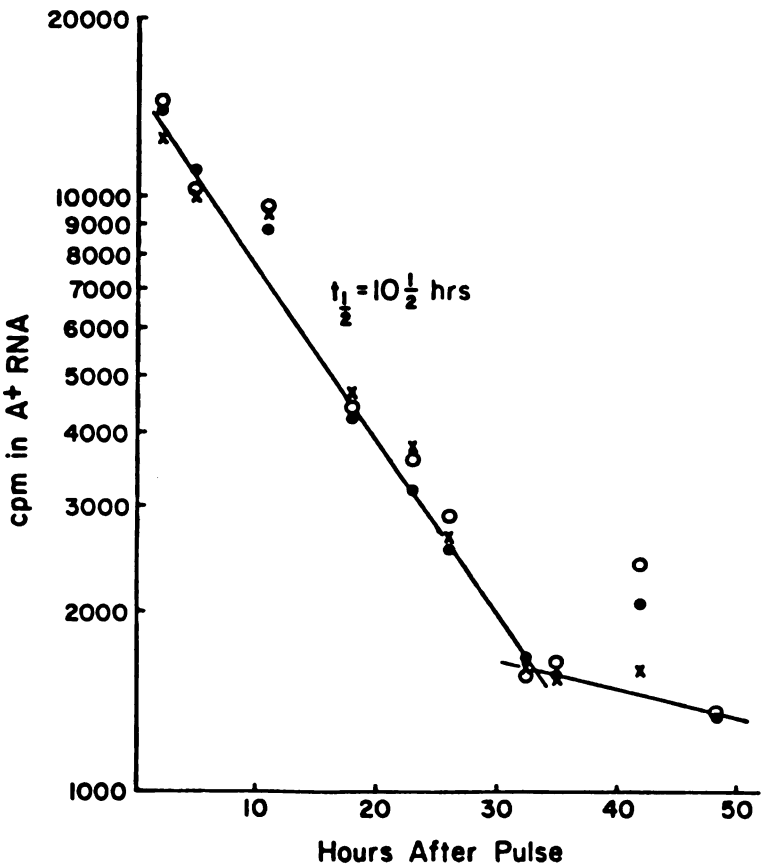

20000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
cpm in A+ RNA
t½ = 10½ hrs
10
20
30
40
50
Hours After Pulse

Figure 4

in the time of appearance of mRNA in the cytoplasm. In order to measure the time of appearance, it was necessary to pre-label the cells for 12 hours with a trace of (^{14}C)-uridine as an internal standard, to correct for differential recovery of the mRNA through the extraction and oligo(dT)-cellulose steps. Cells were treated with 0 or 500 μM STH, then labeled with (5,6 ^{3}H)-uridine, and cytoplasmic RNA was isolated and chromatographed twice on oligo(dT)-cellulose (Figure 5). In control cells the poly(A)$^{+}$ RNA appeared in the cytoplasm after about 20 minutes, which is in agreement with other reports (Perry and Kelley, 1973; Johnson et al., 1975). This was determined by extrapolation of the linear portion of the curve to the time axis. Poly(A)$^{+}$ RNA from cells treated with the inhibitor appeared in the cytoplasm at about 28 minutes, representing approximately a 40% increase in transit time from the nucleus to the cytoplasm. This result was reproduced in several experiments. Poly(A)$^{-}$ RNA, on the other hand, appeared in the cytoplasm at exactly the same time in STH treated and control cells. This argues that the lag in appearance of poly(A)$^{+}$ RNA is not due to failure of STH-treated cells to rapidly take up uridine nor is it due to a general inhibition of RNA metabolism. The slope of the line representing accumulation of (^{3}H)-uridine in poly(A)$^{+}$ RNA was less in STH treated cells, perhaps reflecting some inhibition of mRNA synthesis or destabilization of RNA in the nucleus.

DISCUSSION

We have demonstrated the potency of STH as an inhibitor of mRNA methylation in HeLa cells. The incorporation of methyl groups into poly(A)$^{+}$ RNA was reduced more than the incorporation of radioac-

Figure 5

Time of cytoplasmic appearance of RNA

HeLa cells at 4 x 10^5 per ml were prelabeled with 220 μM (^{14}C)-uridine for 12 hours and adjusted to 2.5 x 10^6/ml, treated with 0 (●-●) or 500 μM STH (o-o) for 1.5 hours and labeled with 4 μM (5,6-^{3}H)-uridine, without removing the STH. At various times after addition of (^{3}H)-uridine, 3.75 x 10^6 cells were removed and placed immediately into ice-cold phosphate buffered saline. Cytoplasmic poly(A)$^+$ RNA and poly(A)$^-$ RNA were prepared subsequently. The ratio of (^{3}H)-uridine to (^{14}C)-uridine in cytoplasmic poly(A)$^+$ RNA and poly(A)$^-$ RNA is presented.

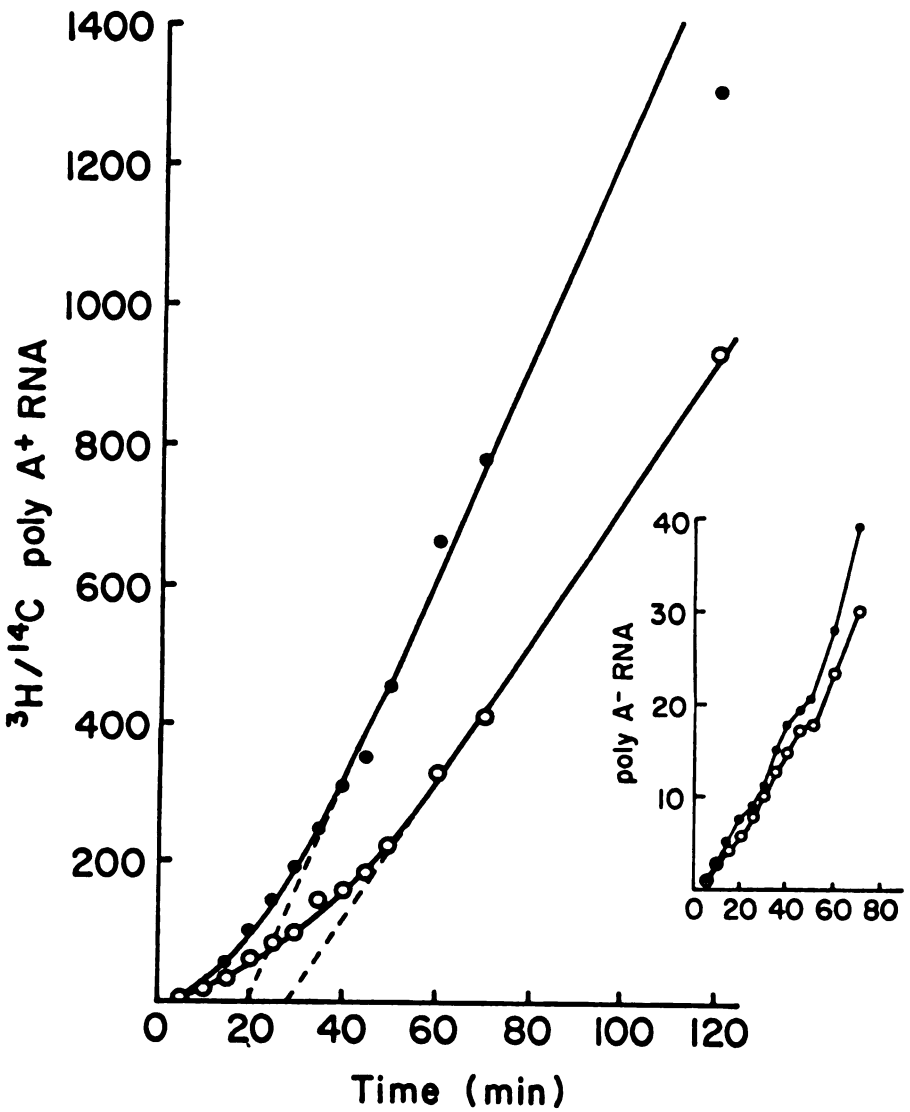

1400
1200
1000
800
600
400
200
$^3H/^{14}C$ poly A+ RNA
0
20
40
60
80
100
120
Time (min)
40
30
20
10
poly A- RNA
0 20 40 60 80

Figure 5

tive uridine (Figure 2), suggesting an overall inhibition of mRNA methylation. Cell viability and protein synthesis were not affected by concentrations of STH which reduced methylation significantly, indicating STH is non-toxic.

We utilized an improved HPLC method for quantitative analysis of the methylated components in labeled mRNA (Figure 3). This method separates all methylated and unmodified nucleosides and cap cores cleanly in a single step. Analysis of (^{3}H-methyl)-methionine labeled RNA by HPLC documented a striking inhibition of m^6A both internally and at the N' position of the cap (80-90%). 2'-O-methylation was effected to a lesser degree by STH (50-60%). These experiments provided the framework for utilizing the non-toxic compound, STH, to perturb m^6A methylation and 2'-O-methylation of HeLa cell mRNA and thereby analyze the role of these modifications in mRNA metabolism.

Our study revealed no change in cytoplasmic mRNA half-life in cultures treated with 50 or 500 μM STH, concentrations which dramatically affected methylation (Figure 4). However, an effect of m^6A on mRNA stability can not be ruled out in experiments where the entire population of mRNA is studied in bulk. A range of half-lives are represented; thus the effect of undermethylation on short half-lived mRNA, for instance, could be masked. It is apparent, however, that undermethylation does not result in dramatic destabilization of the bulk of cytoplasmic mRNA. Since 2'-O-methylation was not as dramatically inhibited by STH as m^6A was, it is difficult to ascertain whether ribose methylation effects mRNA stability.

There are examples in several systems where a change in gene

expression results, at least in part, from alteration of mRNA half-life (Guyette et al., 1979; Heintz et al., 1983; Stiles et al., 1976; Bastos et al., 1977; Chung et al., 1981; Greenberg et al., 1972; Wiskocil, 1980). The mechanism whereby this is accomplished is unknown. Herpes virus shuts off host m^6A methylation late in infection (Bartkoski and Roizman, 1978). Perhaps this could be a mechanism for destabilizing host mRNA. The herpes virus late mRNA transcripts also lack m^6A, and turnover much more rapidly than early transcripts. Not much data is available concerning the methylation of specific host-cell mRNAs. Globin, a mRNA with a relatively long half-life, is thought not to contain m^6A, whereas histone, a nonpolyadenylated mRNA with a short half-life, also does not contain m^6A (for references see Banerjee, 1980). These particular mRNAs could be special cases, or individual mRNAs could have different sensitivities to changes in methylation. It will be important to focus on some specific mRNAs to determine if their stabilities are altered in STH treated cells.

Methylation of m^6A and 2'-O-methylation of ribose are not essential for processing or transport of many mRNA molecules because undermethylated mRNA is found in the cytoplasm. To determine whether methylation is important for the efficiency of events in nuclear mRNA maturation such as splicing, polyadenylation, or transport, we decided to measure the time and rate of accumulation of mRNA in the cytoplasm of HeLa cells treated with 0 or 500 μM STH. If methylation is required for any of the nuclear processing events to take place efficiently, a delay in the time of appearance would be expected. If STH only affected mRNA synthesis or nuclear stability, a change in the

rate of appearance would be observed. The results indicated that normally mRNA appears in the cytoplasm after 20 minutes, whereas the undermethylated mRNA did not appear until about 28 minutes, representing a dramatic increase in the time a mRNA dwells in the nucleus. The slopes of the linear portion of the curves (Figure 5) were more different than would be expected from the amount of inhibition of mRNA synthesis observed in (^{14}C)-uridine labeling experiments (Figure 2), perhaps reflecting an enhanced nuclear turnover.

As with any study involving use of inhibitors, interpretations must be made with caution since many cellular processes may be affected by alterations in available SAM and SAH. The importance of polyamines in rRNA processing is an important example. Early studies with methionine deprivation or ethionine treatment of HeLa cells implicated the involvement of methylation in processing 32S rRNA to 28S and in nuclear RNA stability (Vaughan et al., 1967; Wolf and Schlessinger, 1977). However, subsequent studies with temperature sensitive mutants in conversion of 32 S to 28 S rRNA revealed a defect in polyamine biosynthesis (Levin and Clark, 1979). This does not rule out an effect of methylation on rRNA processing, but suggests that use of methylation inhibitors with pleiotropic effects could lead to erroneous conclusions. In this regard, it is important to note that STH is not a potent inhibitor of polyamine biosynthesis. In tissue homogenates, STH produced less than a 20% reduction in spermine and spermidine synthetase activities when present at 1 mM concentration (Hibasami et al., 1980). Thus, we do not think that the effects we are observing in the presence of STH are due to perturbation of polyamine

synthesis.

In conclusion, our studies have demonstrated the utility of STH as a potent, non-toxic inhibitor of mRNA methylation in HeLa cells, especially m^6A methylation, and have set the stage for analyzing the role of methylation in the metabolism of specific HeLa cell mRNAs. The abundance of undermethylated mRNA in the cytoplasm revealed that methylation is not required for maturation of the mRNA in the nucleus and export to the cytoplasm. The unaltered turnover of undermethylated RNA indicates that the influence of methylation on cytoplasmic stability, if it exists, is subtle, restricted to rapidly turning over RNA or probably important only for a select group of mRNA molecules. The altered time and rate of appearance of undermethylated mRNA in the cytoplasm of STH-treated cells suggests a role for methylation in efficient processing and transport of the bulk of mRNA transcripts. Perhaps transcripts deficient in m^6A are inefficiently or inaccurately processed leading to enhanced nuclear turnover. These possibilities are being explored using cloned DNA probes to study the nuclear processing of specific mRNA transcripts in the presence and absence of STH.

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