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Evidence from UV transcription mapping in HeLa cells that heterogeneous nuclear RNA is the messenger RNA precursor

(primary RNA transcripts/transcription units/mRNA formation/UV irradiation/adenovirus type 2)

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ABSTRACT **The effects of UV irradiation on the incorporation of [^{3}H]uridine in HeLa (human) cell mRNA, rRNA, heterogeneous nuclear RNA (hnRNA) and early mRNA from adenovirus type 2 have been compared. The UV target size of cell mRNA is at least 3 times larger than the average size of the mRNA itself and larger than the adenovirus-2 early mRNA, which is known to derive from transcription units of about 1.5–5.0 kilobases. The UV target size of hnRNA, in contrast, is about the same as its size determined by sedimentation and overlaps with the target size of mRNA. It is concluded that most mRNA derives from a higher molecular weight hnRNA molecule.**

Sauerbier and his colleagues have demonstrated (1–5) that ultraviolet irradiation of cells interrupts RNA transcription in a defined manner: pyrimidine dimers, which form randomly in DNA as a result of irradiation, apparently cause premature chain termination without affecting initiation. Promoter-proximal transcription is thus affected less than promoter-distal transcription (1–4). Moreover, within a given transcription unit there is an exponentially increasing chance of a UV lesion in proportion to the distance from the promoter. Thus, the distance between a given gene and its promoter can be measured by "UV transcription mapping." First applied to bacterial rRNA and bacteriophage mRNA production (1–3), UV transcription mapping has been successfully used to define the transcriptional units (transcription target sizes) of L-cell rRNA (4) and heterogeneous nuclear RNA (hnRNA) (5), vesicular stomatitis virus mRNAs (6), adenovirus type 2 (AD-2) nuclear RNA (7), and AD-2 mRNAs late in infection.*

We have investigated whether UV transcription mapping can define the primary transcript size for HeLa cell mRNA by comparing the target size of pulse-labeled poly(A)-containing cytoplasmic RNA with that of total hnRNA and rRNA. As a control for other effects of UV irradiation on HeLa cell mRNA, a comparison was also made with early AD-2 mRNA formed from transcription units, the sizes of which have been independently measured by other methods to be between about 1.5 and 4.5 kilobases (kb) (8). The data suggest an average target size for cell mRNA of 4–7 kb, whereas the length distribution of the mRNA itself averages ~1.5 kb (9). The transcription unit size of hnRNA, earlier shown by pulse-labeling techniques to have a median of 5–10 kb, with a range of approximately 2–20 kb, is confirmed by UV target size. Thus, the cell mRNA target size and hnRNA transcription unit size cover approximately the same ranges.

MATERIALS AND METHODS

The growth and labeling of both virus-infected and uninfected HeLa (human) cells have been described, as has the preparation of hnRNA, cytoplasmic poly(A)-containing RNA [poly(A)$^+$ mRNA] (7–11). Radioactivity in 18S, 28S, and 4 + 5S RNAs was assayed by sedimentation analysis of material not bound by poly(U)-Sepharose.

UV irradiation of cells was performed as previously described (7) with minor modifications. Briefly, growing cells or cells that had been infected with AD-2 virus at 1000–2000 plaque-forming units per cell for 3 hr were collected by centrifugation at 37°, resuspended in Eagle's medium (12) supplemented with 5% fetal calf serum without vitamins or phenol red and irradiated in 150-mm petri dishes in 25-ml portions. Labeling took place 20 min later. In experiments in which pulse-labeled hnRNA was measured, actinomycin D at 0.04 μg/ml was added during this time.

Hybridization assays for virus-specific RNA were carried out on nitrocellulose filters as described (7). In some experiments equal amounts of ^{32}P-labeled cells were added to all samples prior to RNA extraction as a control for possible unequal efficiencies of RNA extraction and poly(U)-Sepharose selection, but this proved unnecessary and was therefore not included in every experiment.

RESULTS

Target size of HeLa cell mRNA formation

Identical samples of HeLa cells were concentrated in Eagle's medium lacking neutral red and exposed to various doses of UV light or not exposed at all, and 20 min was allowed for the cells to establish a new equilibrium state of transcription. [^{3}H]Uridine was added for 50 min and mRNA formation was assayed by measuring the label in cytoplasmic poly(A)$^+$ RNA in the cytoplasmic postmitochondrial supernatant. The radioactivity recovered in poly(A)$^+$ RNA from irradiated cells was compared to the radioactivity recovered in the same fraction of unirradiated control cells and the % survival of mRNA labeling was calculated. The UV sensitivity of the labeling of 18S and 28S rRNA present in the RNA fraction not bound to poly(U)-Sepharose was also determined. In addition to the irradiation experiments with growing cells, the UV sensitivity of incorporation of label into the poly(A)$^+$ RNA was also determined in cells treated during the 20 min after UV irradiation with actinomycin at 0.04 μg/ml to halt ribosomal RNA synthesis.

The results of a typical experiment are plotted in Fig. 1 *left*. The same results were obtained in several other experiments. The surviving fraction of labeled poly(A)$^+$ RNA decreased as UV dose increased with an average sensitivity slightly greater than that of 18S RNA but less than that of 28S rRNA. In the majority of experiments the slope of decrease in mRNA labeling

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Abbreviations: hnRNA, heterogeneous nuclear RNA; AD-2, adenovirus type 2; kb, kilobases (1000 nucleotides); poly(A)$^+$ RNA, RNA with 3′ poly(A).

* S. Goldberg, J. Nevins, and J. E. Darnell, Jr., unpublished data.

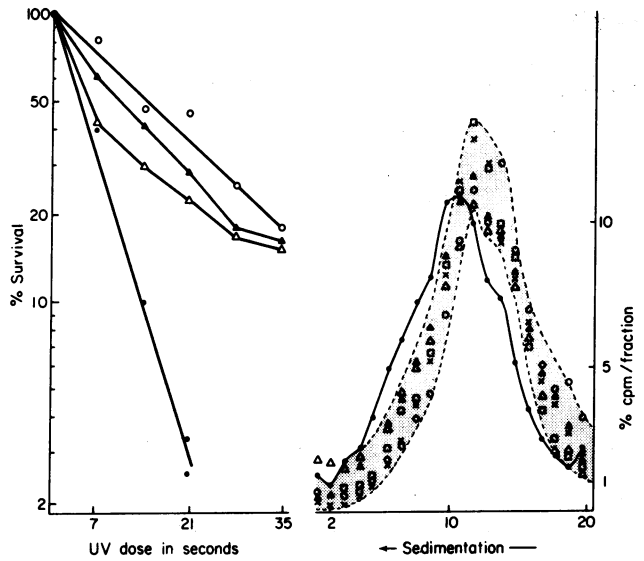

FIG. 1. Effect of UV irradiation on labeling of mRNA and rRNA in HeLa cells. (*Left*) HeLa cells were collected at 37°, washed twice, and resuspended at 3×10^5/ml in the medium without phenol red. Twenty-five-milliliter samples were placed in 150-mm petri dishes and exposed at 37° for 0, 7, 14, 21, 28, or 35 sec to UV light at 254 nm; energy fluence rate was 40 ergs/mm^2 per sec. To half of each sample actinomycin D (0.04 μg/ml) was added for 20 min and then each sample was labeled with [^{3}H]uridine (1 mCi; 20 mCi/μmol). The poly(A)-terminated cytoplasmic RNA was selected from each sample and the % survival of label incorporation was calculated. The 28S and 18S rRNA were assayed by zonal sedimentation of the poly(U)-Sepharose-unbound fraction of the samples not treated with actinomycin D and survival was calculated: ○, 18S; ●, 28S; △, mRNA; from actinomycin-treated cells; ▲, mRNA from growing cells. (*Right*) Samples of poly(A)-terminated cytoplasmic RNA from the experiment described above were sedimented (with 28S and 18S markers in a separate gradient). Percent of total cpm recovered is plotted for each sample. No irradiation, ●—●; 7, 14, 21, 28, 35 sec of UV, other symbols.

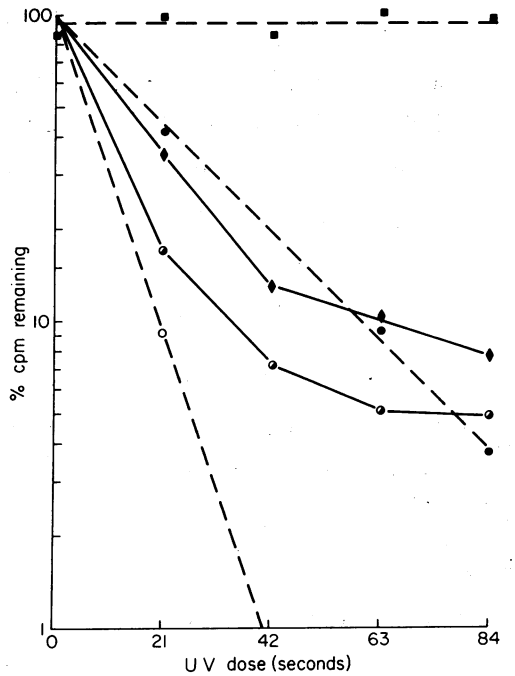

FIG. 2. HeLa cells were infected for 3 hr with AD-2 (2000 particles/cell; see ref. 7), washed twice, and resuspended in medium without phenol red, and aliquots were irradiated for 0, 21, 42, 63, and 84 sec (see Fig. 1). Twenty minutes later each aliquot was labeled for 45 min with 2.5 mCi of [^{3}H]uridine (20 mCi/μmol). Cytoplasmic RNA was isolated and fractionated into poly(A)$^+$ and poly(A)$^-$ positions. The poly(A)$^+$ RNA was also assayed for radioactivity in AD-2-specific hybrids (about 10% of the total in the control sample). The poly(A)$^-$ RNA was separated into 18S, 28S, and 4 plus 5S by zonal sedimentation. Plotted is the relative UV inactivation of each of these categories of cytoplasmic RNA: ■- - -■, 4S + 5S; ●- - -●, 18S; ◑- - -◑, 28S; ○- - -○, total cytoplasmic poly(A)$^+$ RNA; ♦—♦, total AD-2-specific cytoplasmic poly(A)$^+$ RNA.

decreased with increasing dose (i.e., the curve appeared multicomponent), indicating a range of different sizes of the primary target of the UV damage. The 18S rRNA lies around 5 kb and that of 28S rRNA around 11 kb from the origin of RNA synthesis as determined by electron microscopic (13) and UV transcriptional mapping (4). Thus, the average target size of the poly(A)$^+$ cytoplasmic RNA between 5 and 10 kb is considerably larger than the 1.5 kb found to be the average size of HeLa cell mRNA (9).

To confirm that the poly(A)$^+$ RNA had the size distribution characteristic of mRNA, zonal sedimentation analysis of the RNA was performed (Fig. 1 *right*). The absence of peaks at positions corresponding to 28 S, 18 S, or 4 S signifies the absence of ribosomal rRNA and the maximum size about 18 S indicates freedom from hnRNA contamination. A similar result was obtained for the sedimentation pattern of labeled poly(A)$^+$ RNA from cells not treated with the low dose of actinomycin D (data not shown). Because over 75% of the total poly(A)$^+$ cytoplasmic RNA is in polyribosomes (11) the poly(A)$^+$ fraction will be referred to as mRNA. The sedimentation profile in Fig. 1 *right* was plotted as % cpm recovered in each fraction, which demonstrates that the overall sedimentation pattern of the labeled mRNA was very little affected by UV irradiation. A slight shift to smaller average mRNA size occurred in the sample irradiated for the lowest dose (35% depression of labeling), but the profiles were indistinguishable after four higher doses, the highest of which decreased the total labeling by 82%. Thus, the UV target size of mRNA *does not* strictly correspond to its length. This contrasts with previous results obtained for poly(A)-containing hnRNA (5).

Target size of AD-2 early mRNA

To test the possibility that the UV-mediated decrease of mRNA labeling was simply due to a nonspecific depression of mRNA synthesis unrelated to the target size of primary transcripts for mRNA, it was necessary to show that at least some type of mRNA has a different target size. An experiment was designed to take advantage of new information about the transcription units of the DNA-containing virus AD-2 (7, 8, *). During infection with this virus, the DNA enters the cell nucleus, where a number of virus-specific mRNAs are formed early in infection (14) from relatively short (~1.5 to ~4 or 5 kb) transcription units; (8) cellular mRNA is still synthesized and transported to the cytoplasm at this time of infection. Three hours after infection with AD-2, HeLa cells were irradiated and labeled for 45 min and the total mRNA (only about 5% of which was virus-specific) was measured by poly(U)-Sepharose selection. The virus-specific mRNA was measured by hybridization to AD-2 DNA. In addition, new ribosomes are still made at this stage of infection (15) and therefore the labeling of cytoplasmic rRNA after UV could also be measured. The results (Fig. 2) showed that again cell mRNA had a higher UV sensitivity than 18S rRNA, while the majority of AD-2 mRNA, which comes from short transcription units, had a lesser UV sensitivity than did the cell mRNA. In experiments with specific AD-2 early mRNAs to be reported elsewhere (S. Goldberg, M. Wilson, and

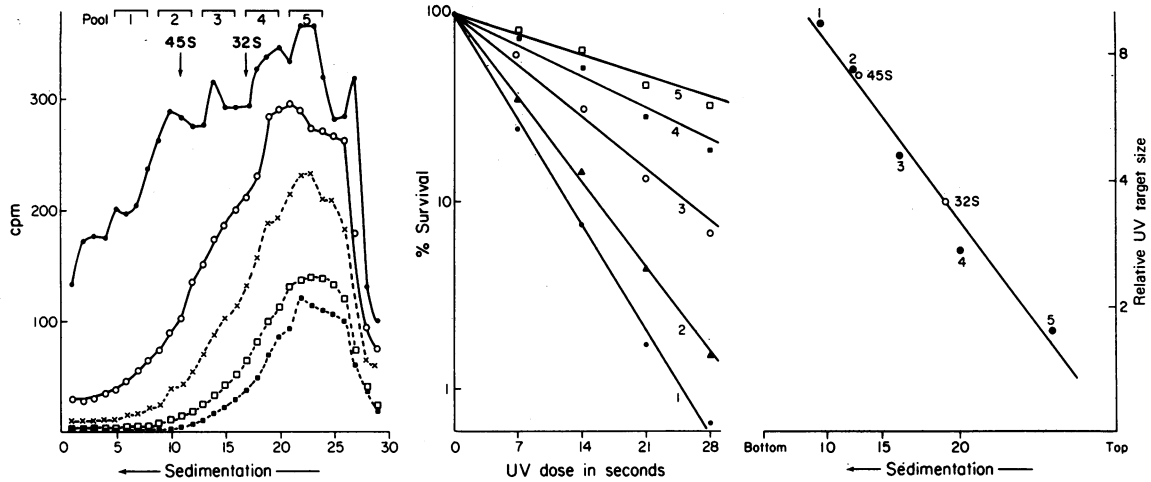

FIG. 3. (*Left*) HeLa cells were labeled after UV irradiation (see Fig. 1) with 5 mCi of [^{3}H]uridine (20 mCi/μmol) for 30 sec. The incorporation was terminated by pouring the cells over frozen phosphate-buffered saline. In the cell fractionation the detergent wash (16) was not performed prior to hnRNA extraction (9, 10). Ethanol-precipitated RNA was dissolved in 80% (vol/vol) dimethyl sulfoxide, heated 2 min at 65°, diluted with 2 volumes of buffer containing 0.01 M EDTA, 0.01 M Tris·HCl at pH 7.4, and 0.2% sodium dodecyl sulfate, and centrifuged as described (9). ●—●, Control; ○—○, 7 sec; ×- - -×, 14 sec; □- - -□, 21 sec; ■- - -■, 28 sec. (*Center*) The cpm sedimenting in regions 1–5 of the control gradient and each of the irradiated samples were summed and the inhibition of cpm in each of these regions of the gradient was plotted with respect to UV dose. The numbers adjacent to each line represent the region of each gradient plotted. (*Right*) The inactivation slope of each region of the gradient (UV target sizes) was plotted against gradient fraction number. The known lengths of marker 45S and 32S pre-rRNA were also plotted in arbitrary units on the same graph in order to define the relationship between molecular weight and distance sedimented in this gradient.

J. E. Darnell), the shortest early transcription unit [which maps in the 0–4.4 region of the genome (14)] had one-third the UV sensitivity of a larger transcription unit [which maps between 73 and 65 on the physical map (8) and reads in the leftward direction (see ref. 14 for map)]. The conclusion from these results and Fig. 2 is that UV transcription mapping is applicable to cell mRNA, the average target size being 5–7 kb.

Correspondence of UV target size and sedimentation size for hnRNA

A central unanswered question in mRNA biosynthesis is whether the hnRNA is a precursor to mRNA. The findings in the previous section—that the UV target size of mRNA is apparently larger than the mRNA itself and that the mRNA target size does not correlate with its length—allow the possibility that most mRNA is derived from hnRNA. Experiments were recently described (10) in which the size distribution of the total nascent labeled hnRNA population was used to predict the distribution of transcription units in HeLa cells. This analysis was extended by examining in detail the UV sensitivity of label incorporation into nascent hnRNA. A quantitative analysis of these experiments (given in Fig. 3) reveals that, in contrast to mRNA, different chain sizes of hnRNA have different UV target sizes. In addition, the nascent chain sizes of hnRNA average between 5 and 10 kb by the UV mapping techniques as well as sedimentation analysis of the nascent labeled hnRNA.

Fig. 3 shows the sedimentation analysis of HeLa cell hnRNA taken from cells labeled for 30 sec; the RNA was extracted from isolated nuclei, omitting the step of detergent wash (16), which has routinely been employed to rid the nucleus of the last 10–15% of cytoplasmic contamination. The recovery of pulse-labeled hnRNA was somewhat greater omitting this step and the molecules consistently had a slightly higher sedimentation value. Because virtually no labeled RNA other than tRNA and 5S RNA is present in the cytoplasm after very brief label times, the technique of nuclear extraction without detergent wash was used for the irradiation experiments. The estimated transcription unit distribution from an analysis of nascent chain sedimentation profile of Fig. 3 (described in detail in ref. 10) indicated that about 50% of the transcription units were longer than ~7 kb (32 S) and 15% were longer than ~14 kb (45 S). If each sedimentation class observed in nascent hnRNA represents growing, unprocessed molecules, then each successively larger size class should have a greater UV sensitivity. This was assayed in the experiment of Fig 3. Cells were irradiated and incubated for 20 min to allow re-establishment of hnRNA synthesis on the UV-damaged templates. A low dose of actinomycin D was included during the incubation to stop pre-rRNA synthesis. The cultures were then pulse-labeled for 30 sec with [^{3}H]uridine and the sedimentation profile of hnRNA was analyzed. There was a much greater depression by UV irradiation of incorporation into the highest molecular weight (fastest sedimenting) fraction, with a decreasing effect in each succeeding lower molecular weight fraction. Qualitatively this result indicates that both long and short hnRNA molecules are predominantly primary transcripts. A quantitative analysis of the effect of UV on incorporation into nascent hnRNA molecules of various lengths confirms this interpretation.

After a UV dose of d lesions/base pair, all nascent chains of a given length l_0 will have a UV sensitivity of e^{-kl_0d}, because all derive from promoters l_0 bases away. After a label time where only nascent chains are labeled, the cpm in chains of length l_0 is proportional to the total number of chains which will ever achieve or exceed length l_0 (10). This proportionality between labeled nascent chains and transcription unit size will remain true even after UV irradiation. Because only nascent chains are labeled, no new truncated transcripts can possibly appear due to UV action. Thus, the incorporation of radioactivity that survives UV irradiation in nascent RNA of any chain length should decrease simply as e^{-kl_0d}. Furthermore, if the majority of nascent hnRNA is not cleaved during transcription, the UV target size of nascent hnRNA of a particular length should equal the length of that RNA as determined by sedimentation analysis. This prediction is fully confirmed by the analyses shown in Fig. 3 *center* and *right*: (*i*) a semi-log plot of

the UV sensitivity versus UV dose of different size classes of hnRNA shows each RNA size class to behave as a single group of primary transcripts with a single slope of UV sensitivity; and (*ii*) a log–log plot of the relationship between UV sensitivity of labeling of RNA in any particular gradient fraction against fraction number (distance sedimented) in the gradient indicates that the UV target size is proportional to the sedimentation rate. For hnRNA samples of various sizes the slope of log(UV target size) versus log(sedimentation rate) was paralleled by the slope of the log(molecular weight) of 45S and 32S markers (detected by absorbance in the same gradients). Because the UV target size of any particular hnRNA is directly proportional to the size of the RNA as measured by sedimentation, the simplest explanation of this result is that the constant of proportionality is 1, i.e., that the UV target size is equal to the molecular weight estimated by sedimentation. Similar results have been obtained for hnRNA from L cells labeled for 20 min, although the longer labeling time in those experiments introduced additional assumptions not present in the nascent chain analysis (5). It is important to realize, however, that while each hnRNA class behaves as primary transcripts, the distribution of transcript sizes shows only about 20–30% of the total to exceed 10 kb and about 50% to exceed 6–8 kb in length (ref. 10 and analysis of data in Fig. 3).

DISCUSSION AND CONCLUSION

This report examines the effect of UV irradiation on mRNA formation in order to determine whether mRNA is a direct transcription product. The results suggest that, on the contrary, the primary transcription product for most mRNA is a larger molecule: (*i*) the mRNA has a UV target size about 3 times larger than its final size; (*ii*) mRNA, regardless of sedimentation rate, seems to have approximately the same UV target size; (*iii*) in contrast to mRNA, the hnRNA has a UV target size that varies according to its length. An estimate of the transcription unit distribution of hnRNA by both sedimentation and UV target size analysis of nascent RNA shows a predominance of molecules in the same size range as the estimated target size for mRNA. Thus, the majority of mRNA molecules would appear to derive from hnRNA molecules with lengths at least 2 to 3 times the length of the mRNA itself.

A formal possibility, difficult to rule out, proposes the existence of larger molecules that are not the material precursors of mRNA but whose synthesis is necessary for the production of the shorter mRNA molecules. Several types of experiments, however, encourage us to interpret the UV data as an indication of a direct relationship between hnRNA and mRNA. First of all, the UV target size of early AD-2 mRNA is less than the average size for HeLa cell mRNA and it is known that the transcription units for these early AD-2 mRNAs are shorter than 5 kb (8). Furthermore, the different early AD-2 mRNAs themselves have target sizes that vary by about a factor of three (S. Goldberg, M. Wilson, and J. E. Darnell, unpublished data). Second, late in AD-2 infection a large transcriptional unit >25 kb in length has been identified by both pulse labeling (8, 17, 18) and UV transcription mapping (7) to be the major, if not the only, RNA formed from the right-hand 85% of the AD-2 genome. At least five mRNA molecules lie in this region of the genome (14). As in the experiments reported here for hnRNA and mRNA, the labeling of many of the late AD-2 mRNAs derived from this large nuclear transcript has a UV sensitivity similar to the labeling of the very large AD-2 nuclear RNA itself.* Thus, the results with AD-2 nuclear and mRNA synthesis both early and late after infection support the validity of UV transcription mapping of mRNA in demonstrating the size of the primary transcript from which an mRNA is derived.

Recent experiments examining briefly labeled nuclear RNA in erythroleukemia cells and spleen cells reveal (19–21) hemoglobin mRNA-specific sequences in nuclear molecules larger than hemoglobin mRNA. Likewise, sequences of the 15S immunoglobulin mRNA have been identified in a 40S nuclear molecule (R. Wall and M. Gilmore-Hebert, personal communication). These two results also provide evidence in favor of primary transcripts containing mRNA sequences that are larger than mRNA.

Clearly, a general conclusion that hnRNA is the precursor for most mRNA does not predict in individual cases whether a longer primary transcript exists for an mRNA or how much longer it may be. The present results, however, make us believe that most mRNAs will undergo processing including not only terminal additions of a 5′ "cap" and 3′ poly(A) (8), but also cleavage from larger primary transcripts.

Note Added in Proof. R. Giorno and W. Sauerbier have informed us that they examined the UV target size of mRNA in mouse myeloma cells and also conclude that the transcription units are 2 to 4 times as large as the mRNA.

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